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The Bacterial Soft Rots of Certain Vegetables.

H. A. HARDING & W. J. MORSE.

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In Cooperation with New York Agricultural Experiment Station.

BULLETIN No. 147. THE BACTERIAL SOFT ROTS OF CERTAIN VEGETABLES.

PART I. THE MUTUAL RELATIONSHIPS OF THE CAUSAL ORGANISMS.

By H. A. HARDING and W. J. MORSE*

SUMMARY.

1. This is part of an investigation of the soft rots of cabbage, cauliflower and turnip, conducted jointly by the Agricultural Experiment Stations of New York and Vermont, and deals with the morphology and cultural characters of 43 strains of organisms.

2. These strains have been found in connection with the soft rots of a considerable number of the common fleshy vegetables and some of them have been described in literature as separate species.

3. These strains appear to be identical in morphology and a cultural study upon more than 12,000 cultures did not indicate any constant cultural differences aside from the fermentation of sugars.

4. The results from 1,500 fermentation-tube tests indicate that the entire group attacks dextrose, lactose and saccharose with the formation of acid and growth in the closed arm but that the amount of gas normally formed is so close to the amount required to saturate the liquid in the fermentation tube that the appearance of gas in the closed arm is very irregular.

5. On account of this observed variation in gas formation the organisms are arranged in six groups each with its appropriate group number. So far as it has gone this study does not show that more than one species, in the customary meaning of the term, is represented in this collection of organisms but the final word in their classification should be deferred until after a study of the pathogenicity of these cultures.

*During his studies of this problem Prof. Morse was Assistant Botanist of the Vermont Station; he is now Plant Pathologist of the Maine Station.

INTRODUCTION.

Almost every year the attention of plant pathologists is directed toward soft rots of some of the fleshy vegetables and in the aggregate the economic losses from this class of diseases are large. In a number of such diseases the causal organism has been isolated, studied and given a specific name. These rot-producing organisms are often closely related, so closely, in fact, that in working with a freshly isolated pathogen of this class one is usually in doubt as to whether the culture under consideration is a new species or a representative of a species already described.

The attention of one of us (H) was first directed to this group in 1897, in connection with an experimental study of the black rot of cabbage, at the University of Wisconsin. The soft rot of cabbage was observed[1] in the experimental fields and cultures made. When it was found that the organism present in the rotting tissue was distinct in its chromogenesis and other cultural characteristics from *Ps. campestris,* the germ causing the black rot of cabbage, the study of the soft-rot germ was not carried further. In 1898, while studying the distribution of *Ps. campestris* in Europe, a white liquefying organism was again encountered in connection with a soft rot of various members of the turnip family. Since 1899 study of this organism has been in association with Mr. F. C. Stewart, Botanist at the New York Agricultural Experiment Station.

In 1896, Mr. Stewart made successful inoculation experiments using germs isolated from diseased cabbage on Long Island and reproduced the soft rot as it appeared in the cabbage fields. In the following year he likewise determined experimentally that a destructive soft rot of *Amorphophallus simlense* could be reproduced under proper conditions by the inoculation of a pure culture of a species of bacterium which he had isolated from diseased plants of this species. No extended study of these causal organisms was conducted at this time. Since 1900 a comparative study of the bacteria causing

[1] Russell, H. L. and Harding, H. A. A bacterial rot of cabbage and allied plants. Wis. Agr. Exp. Sta. Bul. 65, p. 22. 1898.

the soft rots of cabbage and cauliflower has been conducted at the New York Agricultural Experiment Station. A preliminary report[2] of this work was read before Section G of the American Association for the Advancement of Science at Pittsburg, Pa., June 30, 1902, and printed in *Science,* August 22, 1902.

In 1898 Professor L. R. Jones of the University of Vermont isolated an organism which produced soft rot in carrots and other vegetables and later described this organism under the name of *Bacillus carotovorus.*[3] In the summer of 1899, being convinced that the soft rot of the cabbage was a disease closely allied to carrot rot, as to cause and attendant phenomena, he started a student assistant, Mr. F. R. Pember, on the study of the cabbage rot. Mr. Pember secured three organisms resembling *B. carotovorus* and like that capable of producing a soft rot of vegetables and made comparative studies of them. In 1901 the comparative study, at the University of Vermont, of the morphology and pathogenicity of this group of rot-producing organisms was assigned to one of us (M) and over a score of pathogenic cultures were isolated from various sources. Meanwhile Professor Jones was at work upon the question of the production and activities of the toxic substances and wall-dissolving enzyms which are elaborated by these bacteria.

The fact that these similar lines of investigation were being conducted at these adjoining Stations becoming known, a conference was held between the representatives of the two Stations in July, 1902, and the work of studying the soft rots of these vegetables was divided as follows: The determination of the mutual relationships of the germs involved was assigned to Messrs. Harding and Morse; the study of the enzyms elaborated and their relation to the host plants to

[2] Harding, H. A. and Stewart, F. C. A bacterial soft rot of certain cruciferous plants and *Amorphophallus simlense. Science,* N. S., **16**: 314–315. 1902.

[3] Jones, L. R. *Bacillus carotovorus,* n. sp., die Ursache einer weichen Fäulnis der Möhre. *Centbl. Bakt. u. Par., II,* **7**: 12–21; 61–68. 1901. Also, Jones, L. R. A soft rot of carrot and other vegetables. Ann. Rep. Vt Agr. Exp. Station **13**: 299–332. 1901.

Mr. Jones; while the pathogenicity of the germs and the economic relations of the diseases were left to the activities of both Stations. Later, owing partly to the transfer of Mr. Morse to the Maine Station, it was decided that the work on the pathogenicity should be carried out at the New York Station. It was hoped that the three parts of this investigation might be published together, but this does not seem to be practicable. Accordingly, the present paper deals with the first two divisions, leaving the third to appear later.

The economic importance of this inquiry lies mainly in connection with the rots of carrots, cabbage, cauliflower and turnips. In order more fully to understand the relation of the organisms causing these rots it seemed desirable to include a study of a number of bacterial forms which have been known to cause similar diseases in other cultivated plants. Where these organisms are sufficiently similar as to be able in turn to attack the above vegetables their importance in connection with any rotation of crops is evident.

It is a matter of regret, in view of the close relationship which has been found between pathogens on such a wide range of plants, that the circumstances did not permit a comparative study of all the germs known to cause soft rots of plants. It is evident that such a study would yield results of great value.

The earliest isolation of this class of organisms which has come to our attention was that by Professor Pammel in 1892. His attention had been called to a destructive rot of rutabagas and yellow turnips and he states that he "carefully removed with a sterilized scalpel some of the black areas adjoining healthy tissue with a platinum needle, using the test tube of agar. Several forms of bacteria were obtained, and one of these, a bacillus producing a whitish growth on the surface of the agar, when inoculated into an apparently healthy rutabaga produced rot."[4] These fields were undoubtedly like many which have since come under our observation in that there were two distinct diseases present at the same time. Having later obtained well-marked results from inoculations

[4] Pammel, L. H. Bacteriosis of rutabaga. (*Bacillus campestris*, n. sp.) Iowa Agr. College Exp. Station Bul. 27, p. 133. 1895.

with a yellow organism which he named *Bacillus campestris,* no further work was done with the white organism which, in the light of later experience, appears to have been the more important pathogen.

Strictly speaking, the black rot of cabbage and allied plants, caused by *Bacillus,* or as it is now called, *Pseudomonas campestris,* should be included among the soft rots. While this disease ordinarily manifests itself by a drying and browning of the foliage it also often appears as a soft rot of the fleshy tissue. However, as its causal organism can be easily distinguished from the germs producing the soft rots which we have been studying, we have not included *Ps. campestris* among the organisms discussed in this paper.

As has been already suggested, the prime object of our study was to remove the present confusion which exists as to the relationships of the closely allied, white organisms which cause the soft rots of the carrot, turnip, cabbage and cauliflower.

SOURCES OF OUR CULTURES.

Professor M. C. Potter presented the results of his study of a bacterial disease of turnips to the University of Durham Philosophical Society[5] in December, 1898, in which he named the causal organism *Pseudomonas destructans,* and presented a very similar paper at the British Association[6] meeting at Dover in September, 1899. The published abstracts of these papers characterized this disease as a white rot and included a brief description of *Pseudomonas destructans.* Aside from the pathogenicity, his description in these first papers is so brief that it is very doubtful if his organism could be recognized by its aid. He characterized the organism more fully in a paper read before the Royal Society[7] in December, 1900, and translated later into German.[8] Judging from correspondence

[5] Potter, M. C. On a bacterial disease—white rot—of the turnip. From Univ. of Durham. Philo. Soc. Proc. Nov., 1899.

[6] Potter, M. C. On white rot—a bacterial disease—of the turnip. British Asso. for Adv. of Sci. Report for 1899: 921–922. 1900.

[7] Potter, M. C. On a bacterial disease of the turnip. (*Brassica Napus*). *Royal Soc. of London, Proc.* **67:** 442–459, 1901. Reviewed also in *Ztschr. Pflanzenkr.,* **12:** 170. 1902.

[8] Potter, M. C. Ueber eine Bakterienkrankheit der Rüben (*Brassica Napus*). *Centbl. Bakt. u. Par., II,* **7:** 282–288; 353–362. 1901.

with Prof. Potter, the causal organism had been lost before the autumn of 1902. In February, 1903, he kindly furnished us, for use in this comparative study, a culture which he had freshly isolated and believed to be *Pseudomonas destructans*. However, a flagella stain showed that this was a peritrichie organism and accordingly would be classed, according to Migula, as a bacillus.

The organism which we received can hardly be looked upon as an accidental contamination since it is a typical member of the group producing soft rot in plants and is undoubtedly a true English representative of this widespread group. The failure of Prof. Potter to reisolate a Pseudomonas from decaying vegetables is not surprising in view of the fact that during the years in which we have been frequently making such isolations we have never isolated a white liquefying Pseudomonas producing this decay.

Later we obtained a culture labeled *Pseudomonas destructans* from Kral, but this was possibly derived from the same culture sent us under that name by Prof. Potter as it was likewise a bacillus.

In January, 1909, Prof. Potter kindly furnished us with a third freshly isolated culture of *Pseudomonas destructans*. The arrangement of the flagella on this was also peritrichiate and consequently it should be classed as a bacillus. In this paper the culture furnished us by Prof. Potter in 1903 will be designated as Potter's Bacillus.

Reference has already been made to the publications by Professor Jones on the soft rots of carrot and other vegetables. These publications not only included an extended description of *Bacillus carotovorus,* but also gave the results of extensive tests of its pathogenicity when inoculated into a large number of common vegetables. These results showed that, under proper conditions, soft rots of a long list of common vegetables could be produced by inoculations from a pure culture of this germ. Fortunately the original type culture of *Bacillus carotovorus* has been preserved by Professor Jones and was available for use in this comparative study.

Professor F. C. Harrison presented a paper at the Pittsburg meeting of the American Association for the Advancement of Science, entitled "A preliminary note on a new organism producing rot in cauliflower and allied plants,"[9] in which he briefly described the causal organism which he there named *Bacillus olereaceae*. In a later publication[10] he gave a very full account of the behavior of *Bacillus olereaceae* on different culture media as well as the results of inoculations of pure cultures of this bacillus into many of the common vegetables. As the result of such inoculations he reported soft rot being reproduced in cabbage, cauliflower, turnip, rutabaga, rape, radish, parsnip, carrot, mangel, sugar beet (slight), potato, celery, tomato (both ripe and green), artichoke (Jerusalem), asparagus, horse radish, rhubarb, and onions. When we applied to Professor Harrison in 1902 for a culture of *Bacillus olereaceae* he was unable to furnish it because of the loss of all of his stock, but we were so fortunate as to obtain an authentic culture from one which he had previously furnished to Dr. Erwin F. Smith.

An interesting contribution to this subject was made in 1902 by Dr. A. Spieckermann[11] of the Agricultural Experiment Station at Münster, Germany. He isolated an organism which he found to be able to reproduce a soft rot in a considerable number of the common vegetables when inoculated into them in pure culture. The cultural characteristics of this organism differed in some particulars from the ones already described by Potter and Jones, being identical, as will be shown later, with the organism afterwards found and named by Townsend.[12] However, Spieckermann did not name his organism, but in January, 1903, he kindly furnished us with a culture which will be referred to in this publication as Spieckermann's Bacillus.

[9] Harrison, F. C. Preliminary note on a new organism producing rot in cauliflower and allied plants. *Science*, N. S., **16**: 152. 1902.

[10] Harrison, F. C. A bacterial disease of cauliflower (*Brassica olereaceae*) and allied plants. Ontario Agr. College Bul. 137. 1904.

[11] Spieckermann, A. Beitrag zur Kenntniss der bakteriellen Wundfäulnis der Kulturpflanzen. *Landw. Jahrb.*, **31**: 155–178. 1902.

[12] Townsend, C. O. A soft rot of the calla lily. U. S. Dept. of Agr., B. P. I., Bul. 60. 1904.

On May 21, 1902, C. J. J. van Hall presented a doctorate thesis to the University of Amsterdam, entitled "Bijdragen tot de kennis der Bakterieele Plantenziekten,"[13] in which he included an extended description of an organism producing a soft rot in the Iris family and designated by him as *Bacillus omnivorus* n. sp. Dr. van Hall later published a discussion of the pathogenicity[14] of *Bacillus omnivorus* and its manner of attack upon the iris bulbs and plants. While the soft rot of the iris was outside of the group which we originally intended to study we obtained a culture of *Bacillus omnivorus* from Kral and included it among the organisms studied.

In 1904 Dr. C. O. Townsend[15] published a description of a soft rot of calla lily including that of the causal organism which he named *Bacillus aroideae.* Through the kindness of Dr. Townsend we were given a culture of this organism at the very beginning of our work and have made a comparative study of it in connection with the germs from the other sources.

In addition to these named cultures we have made a comparative study of 37 others, isolated from various vegetables. Pember A and C were obtained from decaying cabbages by Mr. F. R. Pember in 1899. In the summer and fall of 1901 one of us (M) isolated cultures XXV, XXVI, XXIX and XXXI from decaying early cabbages from the Vermont Experiment Farm garden; cultures XLVIII, XLIX, L, LI, LII, LIV, LV and LVI from a field of late cabbages in South Burlington, Vt., and cultures XCIV, XCV, XCVI, XCVIII, C, CI, CII, CIII from cabbages in a private storage house at Burlington, Vt. In 1903, an organism, designated as Turnip Rot D, was isolated at the Vermont Agricultural Experiment Station laboratory from decaying turnips growing on the same land from which were obtained the cabbage soft rot organisms XXV-XXXI two years previously.

[13] van Hall, C. J. J. Bijdragen tot de kennis der Bakterieele Plantenziekten. Doct. Thesis. Univ. of Amsterdam. 1902.

[14] van Hall, C. J. J. Das Faulen der jungen Schösslinge und Rhizome von *Iris florentina* und *Iris germanica*, verursacht durch *Bacillus omnivorus* v. Hall und durch einige andere Bakterienarten. *Ztschr. Pflanzenkr.*, **13**: 129–144. 1903.

[15] See footnote 12.

At the New York Agricultural Experiment Station laboratory culture 0.2 e was isolated from a decaying cauliflower from Long Island in November, 1901. Culture 0.2 f was isolated in March, 1902, from a diseased cauliflower plant in the green house. Eleven days previous this cauliflower plant had been carefully inoculated with a pure culture of 0.2 e in order to test the pathogenicity of this culture and the reisolation was made to determine the fact that the disease which had been produced was actually caused by 0.2 e. The inoculation had been made as aseptically as possible and the point of inoculation covered with melted grafting wax. The material for the reisolation was taken from the advancing edge of the diseased area and the plates indicated the presence of a pure culture in the plant tissue.

In 1902 the cultures designated as Miller and Riverhead were isolated from decaying stalks of cabbage which had been planted commercially for the purpose of raising cabbage seed. The stalks were collected as typical examples of the early stages of the destructive rot which causes a heavy loss in the cabbage seed industry on Long Island practically every year and there were two stalks from each of two fields located about 10 miles apart in the eastern portion of the island.

Cultures labeled Miller Stalk 2 No. 1 and Miller Stalk 2 No. 2 were derived from the same diseased stalk while Miller Stalk 3 No. 1, Miller Stalk 3 No. 2 and Miller Stalk 3 No. 3 were likewise isolated at the same time from another similarly diseased cabbage stalk from the same field. Riverhead Stalk 2 No. 1 and Riverhead Stalk 3 No. 1 came from two cabbage stalks out of a second field.

Cultures 0.2 RBe and 0.2 RBi were isolated in November, 1901, from a rutabaga. This was obtained from a private garden near Phelps, N. Y., and the upper portion of the rutabaga was soft and vile smelling. The cultures were prepared from blackened fibro-vascular bundles passing through the apparently sound tissue of the rutabaga. Both cultures were obtained from the same plant.

The cultures Cornell I, III and V were isolated in October,

1904, from two turnips from an experimental field at the Cornell Agricultural Experiment Station, cultures I and III being from the same turnip.

Thus this collection of cultures represents England, Holland, Germany, District of Columbia, Canada, Vermont and widely separated points in the State of New York. They were derived from turnip, iris, rape, calla lily, cauliflower and cabbage.

METHODS OF WORK.

On undertaking this comparative study in July, 1902, the first step was an exchange of cultures which had been isolated at the two Stations and a determination of their cultural characteristics. The media used in this study, with certain exceptions, were prepared in accordance with the suggestions of the report of September, 1897,[16] of the committee of the American Public Health Association on standard laboratory methods. The reaction of the media used at the New York laboratory was uniformly 1.5 per ct. normal acid to phenolphthalein while that used at the Vermont laboratory was the same during the earlier years of the study and later was made neutral to this indicator, as it was found that the organisms grew equally well, if not better, in a neutral medium. In the earlier work at the Vermont laboratory lean beef was used in preparing the media, but the resulting presence of muscle sugar in the broth led to the substitution of Liebig's meat extract. At the New York laboratory this meat extract was uniformly used except on rare occasions when beef was used as a check.

If these variations in the technique at the two laboratories had resulted in discordant results they would have been regrettable, but, as the results will show, such was not the case. On the other hand, the presence of these variations in the technique makes it all the more probable that the results here given can be duplicated in any laboratory where care is given to approximating standard conditions of work.

[16] Report of the committee on standard methods for water analysis. *A. P. H. A. Proc.* (*1897*), **23**: 56-100. 1898.

One of the important items which should never be disregarded in comparative work of this kind is the vigor of culture. Especially when they have been long subjected to artificial conditions in a laboratory it is necessary to put the cultures through a revivifying process, a series consisting of transfers of young cultures through bouillon, gelatin and agar having been used with these germs. Comparative cultures were rarely made in duplicate but almost uniformly in triplicate. When cultures long in stock were tested without previously revivifying, discordant results from the three simultaneous cultures were occasionally obtained, while such comparisons made after the stock culture had been revivified practically always gave more accordant results.

In separating these cultures the main difference is in their ability to ferment the different sugars. Accordingly it was absolutely necessary for accurate results that the broth which was used as the basis of these tests should be free from muscle sugar. The absence of this sugar was determined by testing each lot of broth with *B coli,* and when found to be present the sugar was destroyed by the growth of this organism. By using meat extract it was found possible to prepare bouillon free from this sugar, and all of the broth used at the New York laboratory was in this way made free of muscle sugar.

METHODS OF CLASSIFICATION.

Those familiar with such work will appreciate the difficulty encountered in comparing determinations upon so many organisms scattered over a term of years and made in different laboratories. It is rare that two sub-cultures from the same original will give identical quantitative and qualitative results on a variety of media when tested side by side and when considering the large number of cultures tested at different times and places the slight variations are bewildering. Some system of classification is absolutely essential to progress. A large part of the work and delay in connection with this study was due to the absence of such a well-elaborated system.

The suggestion of Fuller & Johnson[17] that the most stable reactions of bacteria could be printed on a card and the reactions of the culture being studied could be indicated by + or —, as the facts required, was a step in the right direction. As soon as there was a collection of these cards the difficulty of their arrangement became evident and this was met in an ingenious way by Gage and Phelps.[18] They devised a group number which both recorded the more important culture reactions and provided a basis for an orderly arrangement of the cards. The value of these improvements was so evident that on this basis a committee of the Society of American Bacteriologists has worked out an official classification card. While this card is probably not in its finished form it has such marked advantages over any other method of classification which is now available that it has been utilized in presenting the results of the present study.

The Society card consists of three essential parts: (1) A group number (see page 263) along the lines originally suggested by Gage, which records the more important facts regarding an organism and at the same time provides a means of arranging the records so that duplicates can be found readily; (2) A brief characterization (see page 264) which may be filled in by means of + or —, as suggested by Fuller and Johnson, and which serves as a means of further comparing germs with the same group number; and (3) A provision for tersely recording the detailed features of cultures upon the common media as first outlined by Chester[19] (see page 284).

The basis upon which the group number is computed according to the Society card of 1907, is given in Table I.

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[17] Fuller, G. W. and Johnson, Geo. A. On the differentiation and classification of water bacteria. *Jour. Exp. Med.*, 4: 609–626. 1899. Similar article in *A. P. H. A., Proc.* 25: 580–586. 1899.

[18] Gage, S. DeM. and Phelps, E. B. On the classification and identification of bacteria with a description of the card system in use at the Lawrence Experiment Station for records of species. *A. P. H. A., Proc.* 28: 494–505. 1903.

[19] Chester, F. D. A manual of determinative bacteriology. MacMillan. 1901.

TABLE I.

A NUMERICAL SYSTEM OF RECORDING THE SALIENT CHARACTERS OF AN ORGANISM. (GROUP NUMBER.)

100.	Endospores produced
200.	Endospores not produced
10.	Aerobic (Strict)
20.	Facultative anaerobic
30.	Anaerobic (Strict)
1.	Gelatin liquefied
2.	Gelatin not liquefied
0.1	Acid and gas from dextrose
0.2	Acid without gas from dextrose
0.3	No acid from dextrose
0.4	No growth with dextrose
.01	Acid and gas from lactose
.02	Acid without gas from lactose
.03	No acid from lactose
.04	No growth with lactose
.001	Acid and gas from saccharose
.002	Acid without gas from saccharose
.003	No acid from saccharose
.004	No growth with saccharose
.0001	Nitrates reduced with evolution of gas
.0002	Nitrates not reduced
.0003	Nitrates reduced without gas formation
.00001	Fluorescent
.00002	Violet chromogens
.00003	Blue "
.00004	Green "
.00005	Yellow "
.00006	Orange "
.00007	Red "
.00008	Brown "
.00009	Pink "
.00000	Non-chromogenic
.000001	Diastasic action on potato starch, strong
.000002	Diastasic action on potato starch, feeble
.000003	Diastasic action on potato starch, absent
.0000001	Acid and gas from glycerine
.0000002	Acid without gas from glycerine
.0000003	No acid from glycerine
.0000004	No growth with glycerine

The genus, according to the system of Migula, is given its proper symbol, which precedes the number, thus:

BACILLUS COLI (Esch.) Mig.	becomes	B.	222.111102
BACILLUS ALCALIGENES Petr.	"	B.	212.333102
PSEUDOMONAS CAMPESTRIS (Pam.) Sm.	"	Ps.	211.333251
BACTERIUM SUICIDA Mig.	"	Bact.	222.232203

RESULTS OF OUR STUDY.

The results of our attempt to classify these cultures representing four named species and thirty-nine other cultures isolated from diseased vegetables are given in Table II.

TABLE II.—SHOWING THE GROUP NUMBER AND OTHER CLASSIFICATORY FEATURES OF THE GERMS STUDIED.

Group Number	NAME	Morphology						Cultural Features														Biochem. Features																	
		Diameter over 1 μ.	Chains	Endospores	Capsule	Pseudozoogloea	Motile	Gram's stain	Turbid	Pellicle	Sediment	Shining	Dull	Wrinkled	Chromogenic	Round	Proteus-like	Rhizoid	Filamentous	Curled	Surface growth	Needle growth	Moderate	Abundant	Discolored	Starch destroyed	Grows at 37 C.	Grows in Cohn's Sol.	Grows in Uschinsky's Sol.	Gelatin	Casein	Agar	Acid curd	Rennet curd	Casein peptonized	Indol	Nitrates reduced	Fluorescent	Plant pathogen
		Broth										Agar					Gel. plate				Gel. stab.		Potato							Liquefaction			Milk						
B.221.1113022	Bacillus carotovorus		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" oleraceae		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" omnivorus		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XXV		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XXVI		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XXIX		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XXXI		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" L		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" LI		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" LII		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" LV		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XCIV		++			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XCV		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XCVI		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XCVII		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" XCVIII		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" CI		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" CII		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	" CIII		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	Pember A		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	Pember C		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	O. R B e		+			+	+		+	+	+	+				+					+		+						+			+		+				+
B.221.1113022	O. R B i		+			+	+		+	+	+	+				+					+		+						+			+		+				+

B.221.1113022	"	O. 2 f	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	Cornell I	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	Cornell III	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	Cornell V	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	Miller 2 No. 1	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	" 2 No. 2	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	" 3 No. 2	—	+	—	—	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	" 3 No. 3	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	Riverhead 2 No. 1	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	LVI	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1113022	"	Miller 3 No. 1	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1123022	"	X LVIII	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.1213022	"	C	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2113022	"	O. 2 e	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2113022	"	LIV	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2123022	"	Potter's	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2123022	"	Riverhead 3 No. 1	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2223022	"	XLIX	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2223022	"	Turnip Rot D	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2223022	"	Spieckermann's	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	+	+	—	+
B.221.2223022	"	aroideae	—	+	—	—		+	—	+	+	+	+	—	—	—	+	—	—	—	—	+	+	+	+	—	+	+	—	+	+	+	—	+		+	+	+	—	+

The above table presents as concisely as possible the results of observations made under 38 headings. These observations were made upon an average of 25 cultures for each of the 43 germs which were studied, or an average of approximately 1,000 cultures for each heading. Owing to the fact that a single culture would furnish the information given under a number of headings, only about 12,000 cultures are actually represented in this table, although some thousand additional were required in the preliminary cultivations and in the revivifying process.

While the above results are perfectly accordant in all of the items covered by + and — it would be misleading to assume that no apparently discordant observations were made. Every item was determined a number of times, usually in triplicate, and in practically all cases these determinations were made in both laboratories, the more important of them having been determined independently by four different workers.

The importance of revivified cultures has already been emphasized and it was in the determinations which were made previous to the application of this preliminary cultivation that the larger part of the variation was encountered. However, revivication does not remove all of the tendency to vary which is resident in some of the cultures.

In summarizing in the table the results which were not entirely accordant the rule has been followed that a single well-marked positive result was more important than a number of negative ones. Wherever a single positive result was marked as doubtful for any reason and the other observations were uniformly negative the test has been recorded as negative in the table, but attention will be called to such cases in the following remarks.

Pellicle on broth. The pellicle on broth is usually thin and in many of the earlier observations it was recorded as absent. Careful observations on cultures which had not been disturbed showed that the pellicle begins with the formation of floating masses of pseudozooglea which are gradually united into a delicate film. If the culture is even gently shaken these sink

and the pellicle which would have resulted is destroyed and may not be reformed.

While this delicate pellicle may be taken as characteristic for the entire collection of cultures, six members of the B. 221.1113022 group—*B. olereaceae,* CI, 0.2 f, Miller Stalk 3 No. 3, Riverhead Stalk 2 No. 1 and Miller Stalk 3 No. 1—have from time to time shown a pellicle somewhat stronger than the rest, which tends to hold together when shaken. The reasons for this variation have not been more closely determined.

Agar colonies. Townsend in his description of *B. aroideae*[20] laid some stress on the diagnostic value of radiating surface colonies which appeared on lightly seeded agar plates that had been inoculated from fresh cultures and been held at 22° to 35° C. Early in the work some attention was given to this point, particularly at the Vermont laboratory. It was found that on lightly seeded plates these radiating, amoeboid-shaped colonies were usually produced by *B. aroideae,* Spieckermann's Bacillus and Turnip Rot D, lending color to the idea that the absence of gas formation was in some manner correlated with a tendency to the formation of these colonies. However, the formation of these colonies was not restricted to these organisms and on at least one occasion they were formed by 0.2 f, Vermont XLVIII and Turnip Rot D when they were not formed by *B. aroideae* nor Spieckermann's Bacillus, although all five were tested together under parallel conditions of media, temperature and dilution.

At the New York laboratory no systematic study was made of this point partly because the work at the Vermont laboratory indicated that it had no diagnostic value and largely because variations in moisture and temperature effect such marked changes in the colony growths of practically all bacteria. The occasional formation of radiating colonies was observed in connection with a considerable number of the

[20] Townsend, C. O. A soft rot of the calla lily. U. S. Dept. of Agr. B P. I., Bul. 60, pp. 17 and 39. 1904.

forms, but no determination was made of the exact conditions under which they were produced.

Potato discolored. The difficulty with this determination lay not so much in a variation in the cultures as in the different conceptions which various workers had of the meaning of the term. As used in the table it refers to the formation of the nearly white halo which is seen around the margin of the growth with certain species of bacteria. This was not observed with this collection of cultures. On the contrary, the portion of the potato not covered with the luxuriant glistening, creamy growth was ordinarily darkened to a faint brownish gray, the extent of this change varying apparently with the characteristics of the potato itself.

Potato starch destroyed. This item has been added to the classification card recently and the determinations were made at the New York laboratory only. The test was made on sterilized potato cylinders in test tubes upon which cultures were grown for two weeks. These potato cultures were crushed in porcelain mortars and 50 cc. of water added to separate the particles so that the effect of the iodine could be easily seen. The presence of unchanged starch was determined by the gradual addition of a weak solution of iodine in potassium iodide. After a sufficient amount has been added to satisfy some of the other compounds which are present the starch grains are turned to a blue-black. The extent of the reduction of the starch by the bacterial growth is estimated on the basis of similar tests of uninoculated potato cylinders. In none of these cultures was the starch reduction complete, but amylodextrin was formed. (See page 331.) This change was expressed by 2 in the appropriate place in the group number.

For the details of this method of determining this action on the starch, we are indebted to Dr. Erwin F. Smith who assisted us by correspondence and by a demonstration in connection with the meeting of the Society of American Bacteriologists at Baltimore.

Uschinsky's Solution. The advantage of this solution lies

in the definite chemical compounds used in its preparation, but unfortunately the variation in the results obtained from its use is wider than in a majority of the media ordinarily employed. Jones and Townsend found this medium well suited to the forms which they studied earlier, but the use of this medium in the later study at the Vermont laboratory did not result in any visible growth. A test of the entire collection of cultures at the New York laboratory in 1909 likewise failed to produce growth in any case. A study of the commercially pure chemicals which had been obtained from one of the most responsible houses revealed the fact that the compound supposed to be di-basic potassium phosphate was really the mono-basic salt. This produced a marked acidity in the medium and probably inhibited the growth. A redetermination with chemicals which were true to name gave an abundant growth in all cases.

Gelatin liquefaction. All of the cultures in this collection produced an evident liquefaction in gelatin stab in all of the many tests except in the case of one series of cultures made at the New York laboratory in 1905, when the entire collection either liquefied very slowly or entirely failed to do so. Shortly before this date some attention was being given in Europe to the suggestion of van't Hoff[21] of the addition of formalin to gelatin to raise its melting point. The gelatin used in the above tests was of the "Gold Label" quality and had been imported through the regular channels. A discussion of this difficulty in the matter of liquefaction before the Society of American Bacteriologists at their Philadelphia meeting brought out the fact that a number of other workers had had similar experiences. With the substitution of a new supply of gelatin all of the soft-rot cultures again produced liquefaction.

While all of these cultures produced an evident liquefaction of gelatin the rate at which this result was produced varied widely both among the different cultures tested at the same time and among the different tests of a single culture. The cultures known as Cornell I and Cornell V were isolated on

[21] van't Hoff, H. J. Erhöhung des Schmelzpunktes der Nährgelatin mittels Formalin. *Centbl. Bakt. u. Par., I*, **30**: 368. 1901.

practically the same date from diseased turnips in the same field and the qualitative record of these cultures in the above table is identical in all particulars. They were as similar as two subcultures from a single source would be expected to be except in the matter of liquefaction. In parallel cultures made in triplicate at the same time, on gelatin stabs from the same flask of gelatin, from agar slopes treated as nearly alike as possible Cornell I produced large dry pits while Cornell V liquefied practically the entire stabs in three weeks. Again in his description of *B. aroideae,* Townsend called attention to the rapidity of its action, completing the liquefaction in 3—5 days. In our studies the culture which he furnished us varied relatively from one of the more rapid to one of the slowest in the collection. The rate and form of liquefaction appears to vary with what may be termed the general vigor of the culture and more especially with regard to the relation of the culture to oxygen. Unfortunately our knowledge of the factors which influence the cultures in these particulars is extremely fragmentary.

Milk—Acid curd. The distinction between acid and rennet curds is of historical rather than practical value in the study of many bacterial forms. In this group the distinction cannot be made with certainty and different observers have varied accordingly. The formation of the curd is probably due to the joint action of an enzym and of acid. The record was made of acid curd + because there is an evident acid formation preceding the curdling.

Casein peptonized. Here again the various observers differed markedly in their conclusions because the visible digestion of the curd is so slight that it cannot with certainty be differentiated from the shrinking which would result from the action of the acid on the curd. The action of the various cultures on milk was alike and the final record of + was based on the results of milk agar plates prepared after the suggestion of Hastings[22] where the ability of the organisms to attack the casein seems to be clearly brought out.

[22] Hastings, E. G. Milchagar als Medium zur Demonstration der Erzeugung proteolytischer Enzyme. *Centbl. Bakt. u. Par., II,* 10: 384. 1902.

Indol. The production of indol by this collection of cultures is slight in all cases and the various observers have not always considered that the red color produced by the test was sufficient to be considered positive. However in both laboratories series of tests have been considered as sufficiently decided to be classed as positive. It will be noted that this result is not in accord with the record of Townsend[23] with *B. aroideae,* but the disagreement is undoubtedly a matter of interpretation of results rather than a difference in organisms.

Nitrate reduction. It was only in a few instances that the reduction of nitrates was not detected in the standard solutions. In all of these instances succeeding cultures showed the characteristic color reaction. The cultures which failed had not been revivified previous to the test.

Gas formation. As has already been indicated the gas formation from dextrose, lactose and saccharose in the fermentation tube offers a basis for classifying this collection of cultures. Owing to the importance of the observation on this point and to the somewhat conflicting results obtained with some of the cultures, the results of the various determinations are here given in Table III.

[23] Townsend, C. O. loc. cit. p. 32.

TABLE III.—BEHAVIOR OF SOFT ROT BACTERIA IN FERMENTATION TUBES.

Date	Laboratory	Observer	Germ	Group No.	Revivified	Dextrose		Lactose		Saccharose	
						Gas	Per ct.	Gas	Per ct.	Gas	Per ct.
1903			B. omnivorus								
1903	N	P	"	.111	—	+		+		+	
1903	V	M	"	.111	—	+	8	+	15	+	6
1904	V	M	"	.1	+	+	2				
1904	N	P	"	.111	—	+		+		+	
1905	N	P	"	.111	+	+		+		+	
1905	V	S	"	.1	+	+	15				
1903			XXV								
1903	N	P	"	.111	—	+		+		+	
1903	V	M	"	.111	—	+	8	+	7	+	3
1903	V	S	"	.111	—	+	5	+	12	±	10
1903	V	S	"	.1	—					+	6
1904	V	M	"	.1	+	+					
1904	N	P	"	.111	—	+		+		+	
1905	N	P	"	.111	+	+		+		+	
1905	V	S	"	.1	+	+	10				
1905	V	S	"	.1	+	+					
1903	N	P	XXVI	.111	—	+		+		+	
1903	V	M	"	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	N	P	XXIX	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	N	P	XXXI	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1905	N	P	"	.111	+	+		+		+	
1905	V	S	"	.111	+	+	12	+	18	+	8
1903	N	P	L	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	N	P	LI	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	N	P	LII	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	N	P	LV	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	N	P	XCVI	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1903	V	M	Pember C	.111	—	+	18	+	7	+	30
1903	V	S	"	.1	—			±	8		
1903	V	S	"	.111	—	+	13	±	2	+	14
1903	N	P	"	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	
1904	V	M	"	.1	+	+	20				
1905	N	P	"	.111	+	+		+		+	
1905	N	P	Cornell I	.111	+	+		+		+	
1905	V	S	"	.111	+	+	5	+	12	+	5
1905	N	P	Cornell III	.111	+	+		+		+	
1905	V	S	"	.111	+	+		+	15	+	4
1905	N	P	Cornell V	.111	+	+		+		+	
1905	V	S	"	.111	+	+	5	+	15	+	5
1903	N	P	Miller 2 No. 1	.111	—	+		+		+	
1904	N	P	"	.111	—	+		+		+	

TABLE III.—BEHAVIOR OF SOFT ROT BACTERIA IN FERMENTATION TUBES.

Date	Laboratory	Observer	Germ	Group No.	Revivified	Dextrose		Lactose		Saccharose	
						Gas	Per ct.	Gas	Per ct	Gas	Per ct.
1903	N	P	Miller 2 No. 2	.111	—	+	....	+	....	+	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.111	+	+	10	+	10	+	10
1905	N	P	"	.111	+	+	....	+	....	+	....
1902	V	M	B. carotovorus	.111	—	+	....	+	....	+	....
1903	N	P	"	.111	—	+	....	+	....	+	....
1903	V	M	"	.111	—	+	....	+	....	+	....
1903	V	M	"	. 11	—	....	....	+	....	+	....
1904	N	P	"	.212	—	—	....	+	....	—	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.1	+	+	....	....	....	....	....
1903	N	P	B. olereaceae	.112	—	+	....	+	....	—	....
1903	V	M	"	.111	—	+	4	+	10	+	3
1904	N	P	"	.112	—	+	....	+	....	—	....
1904	N	H	"	.211	—	—	....	+	....	+	....
1904	V	M	"	.1	+	±	1	....	....	....	....
1905	V	S	"	.1	+	+	10	....	....	....	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1904	V	M	O. RBe	.2	—	—	....	....	....	....	....
1905	V	S	"	.1	—	±	5	....	....	....	....
1905	V	S	"	.111	+	±	1	+	5	+	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1903	N	P	O. RBi	.111	—	+	....	+	....	+	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.111	+	+	10	+	12	+	5
1905	N	P	"	.111	+	+	....	+	....	+	....
1903	N	P	Riverhead 2 No. 1	.111	—	+	....	+	....	+	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1904	N	H	"	.211	—	—	....	+	....	+	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.111	+	+	8	+	12	+	10
1905	N	P	"	.111	+	+	....	+	....	+	....
1903	N	P	CI	.111	—	+	....	+	....	+	....
1904	V	M	"	.1	+	±	1	....	....	....	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.1	+	±	10	....	....	....	....
1905	V	S	"	.112	+	±	1	+	12	—	....
1903	N	P	XCV	.111	—	+	....	+	....	+	....
1903	V	S	"	.212	—	—	....	+	8	—	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1902	V	M	Pember A	.111	—	+	4	+	8	+	10
1903	V	M	"	.111	—	+	10	+	10	+	16
1903	N	P	"	.112	—	+	....	+	....	—	....
1903	V	M	"	. 1	—	....	....	....	....	±	8
1903	V	S	"	.1	—	±	3	....	....	....	....
1903	V	S	"	.111	—	±	6	+	10	±	4
1904	N	P	"	.112	—	+	....	+	....	—	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.1	+	+	18	....	....	....	....
1903	N	P	LVI	.112	—	+	....	+	....	—	....
1904	N	P	"	.121	—	+	....	—	....	+	....
1902	N	H	0.2 f	.21	—	—	....	+	10	....	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1904	N	H	"	.211	—	—	....	+	....	+	....
1904	V	M	"	.2	+	—	....	....	....	....	....

TABLE III.—BEHAVIOR OF SOFT ROT BACTERIA IN FERMENTATION TUBES.

Date	Laboratory	Observer	Germ	Group No.	Revivified	Dextrose		Lactose		Saccharose	
						Gas	Per ct.	Gas	Per ct.	Gas	Per ct.
1905	V	S	0-2 f	.111	+	+	10	+	10	+	5
1905	N	P	"	.111	+	+	....	+	....	+	....
1906	N	P	"	.1	+	±	....	....	....	....	....
1906	N	P	"	.222	+	—	....	—	....	—	....
1903	N	P	CII	.222	—	—	....	—	....	—	....
1904	N	P	"	.222	—	—	....	—	....	—	....
1904	V	M	"	.1	—	+	1	....	....	....	....
1905	V	S	"	.1	+	+	10	....	....	....	....
1905	V	S	"	.111	+	±	1	+	12	+	4
1905	N	P	"	.111	+	+	....	+	....	+	....
1903	N	P	CIII	.212	—	—	....	+	....	—	....
1903	V	M	"	.212	—	—	....	+	8	—	....
1903	V	M	"	.1	—	....	....	+	5	....	....
1904	V	M	"	.1	+	+	1	....	....	....	....
1904	N	P	"	.212	—	—	....	+	....	—	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.111	+	±	1	+	12	±	5
1903	N	P	XCVIII	.111	—	+	....	+	....	+	....
1903	V	M	"	.222	—	—	....	—	....	—	....
1903	V	S	"	.212	—	—	....	±	8	—	....
1903	V	S	"	.1	—	....	....	+	5	....	....
1904	V	M	"	.1	+	+	1	....	....	....	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.111	+	+	10	+	10	+	5
1903	N	P	XCIV	.211	—	—	....	+	....	+	....
1903	V	M	"	.222	—	—	....	—	....	—	....
1903	V	S	"	.212	—	—	....	+	8	—	....
1903	V	S	"	.1	—	....	....	+	8	....	....
1904	V	M	"	.1	+	+	1	....	....	....	....
1904	N	P	"	.111	—	+	....	+	....	+	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.111	+	+	8	+	10	+	5
1903	N	P	Miller 3 No. 3	.222	—	—	....	—	....	—	....
1904	N	P	"	.222	—	—	....	—	....	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.111	+	+	1	+	8	+	1
1905	N	P	"	.111	+	+	....	+	....	+	....
1903	N	P	Miller 3 No. 2	.222	—	—	....	—	....	—	....
1903	V	M	"	.212	—	—	....	+	5	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1904	N	P	"	.222	—	—	....	—	....	—	....
1905	N	P	"	.111	+	+	....	+	....	+	....
1905	V	S	"	.111	+	±	5	+	4	+	1
1905	V	S	"	.2	+	—	....	....	....	....	....
1903	N	P	Miller 3 No. 1	.222	—	—	....	—	....	—	....
1904	N	P	"	.222	—	—	....	—	....	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.1	+	±	1	....	....	....	....
1905	V	S	"	.112	+	±	1	+	5	—	....
1905	N	P	"	.211	+	—	....	+	....	+	....
1905	N	P	"	.2	+	—	....	....	....	....	....
1906	N	P	"	.22	+	—	....	—	....	....	....
1906	N	P	"	.111	+	+	....	±	....	±	....
1902	V	M	XLVIII	.212	—	—	....	+	8	—	....
1903	N	P	"	.112	—	+	....	+	....	—	....
1903	V	M	"	.222	—	—	....	—	....	—	....
1903	V	M	"	.1	—	....	....	+	2	....	....

TABLE III.—BEHAVIOR OF SOFT ROT BACTERIA IN FERMENTATION TUBES.

Date	Laboratory	Observer	Germ	Group No.	Revivified	Dextrose		Lactose		Saccharose	
						Gas	Per ct.	Gas	Per ct.	Gas	Per ct.
1903	V	M	XLVIII, cont'd	.212	—	—	....	+	9	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1904	N	P	"	.112	—	+	....	+	....	—	....
1905	N	P	"	.212	+	—	....	+	....	—	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.112	+	±	1	+	12	—	....
1905	N	P	"	.2	+	—	....	....	....	....	....
1906	N	P	"	.21	+	—	....	+	....	....	....
1906	N	P	"	.212	+	—	....	±	....	—	....
1903	N	P	C	.121	—	+	....	—	....	+	....
1904	N	P	"	.121	—	+	....	—	....	+	....
1903	N.	P	LIV	.211	—	—	....	+	....	+	....
1904	N	P	"	.211	—	—	....	+	....	+	....
1902	N	H	0.2 e	.21	—	—	....	+	10	....	....
1903	V	M	"	.212	—	—	....	±	1	—	....
1903	V	M	"	.211	—	—	....	+	6	+	4
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.211	+	—	....	+	10	±	2
1905	N	P	"	.211	+	—	....	+	....	+	....
1905	N	P	"	.2	+	—	....	....	....	....	....
1906	N	P	"	.11	+	±	....	+	....	....	....
1906	N	P	"	.1	+	±	....	....	....	....	....
1906	N	P	"	.211	+	—	....	+	....	±	....
1903	V	M	Potter's Bacillus	.212	—	—	....	+	4	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.21	+	—	....	+	10	....	....
1905	N	P	"	.212	+	—	....	+	....	—	....
1905	N	P	"	.2	+	—	....	....	....	....	....
1906	N	P	"	.21	+	—	....	+	....	....	....
1906	N	P	"	.212	+	—	....	±	....	—	....
1903	N	P	Riverhead 3 No. 1	.212	—	—	....	+	....	—	....
1904	N	P	"	.212	—	—	....	+	....	—	....
1904	N	H	"	.222	+	—	....	—	....	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.222	+	—	....	—	....	—	....
1905	N	P	"	.222	+	—	....	—	....	—	....
1903	V	S	B. aroideae	.222	—	—	....	—	....	—	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	N	P	"	.222	+	—	....	—	....	—	....
1905	N	P	"	.2	+	—	....	....	....	....	....
1906	N	P	"	.22	+	—	....	—	....	....	....
1903	V	S	Spieckermann Bacillus	.222	—	—	....	—	....	—	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	N	P	"	.222	+	—	....	—	....	—	....
1905	N	P	"	.2	+	—	....	....	....	....	....
1903	N	P	XLIX	.222	—	—	....	—	....	—	....
1904	N	P	"	.222	—	—	....	—	....	—	....
1903	N	P	Turnip Rot D	.222	—	—	....	—	....	—	....
1903	V	S	"	.222	—	—	....	—	....	—	....
1904	V	M	"	.2	+	—	....	....	....	....	....
1904	N	P	"	.222	—	—	....	—	....	—	....
1904	N	H	"	.222	+	—	....	—	....	—	....
1905	N	P	"	.222	+	—	....	—	....	—	....
1905	V	S	"	.2	+	—	....	....	....	....	....
1905	V	S	"	.2	+	—	....	....	....	....	....

It will be seen from this table that the determinations of fermentative ability extended from 1902 to 1906, but that the larger amount of work on this phase of the subject was done in 1904 and 1905. Under laboratories, V and N refer to the Vermont and New York laboratories respectively. Under observers, M and H refer to the authors while S and P indicate that the actual manipulations of the determinations were done by L. P. Sprague or M. J. Prucha, assistants at the two laboratories. These tests were made under the immediate supervision of the authors, and the results were, for the most part, personally inspected by them, and they accept the responsibility for their accuracy.

Under group number is given the group number which would result from the particular determinations in question and an inspection of this heading under each organism brings out sharply the variations observed.

It should be remembered that each of the 550 determinations of fermentative ability recorded in the above table was conducted almost exclusively in triplicate, a comparatively small number having been made in duplicate so that this table really summarizes the results from approximately 1,500 fermentation tube cultures. It occasionally happens that in a fermentation test in triplicate, gas will not appear in one or more of the tubes and this is especially liable to happen where the total amount of gas is small, as in the case of this group of organisms. A complete record of these discordant results was kept at the Vermont laboratory and the cases are indicated in the table by $\pm$. The total number of these cases observed at the Vermont laboratory was 23. At the New York laboratory where the card system of note keeping was being tried in various forms the record on the card was made on the basis of the triplicate test rather than that of the individual fermentation tube and the exact number of these variations is not known, but they were probably not greater than those given for the Vermont laboratory.

In the fermentation tubes which were used the closed arm has a capacity of 10 cc. In all but a few cases the gas produced had a volume of 1 cc. or less and accordingly was re-

corded as 10 per ct. or less. Owing to the rounded form of the upper end of the fermentation tube it is difficult to estimate quantities under 10 per ct. with any considerable accuracy. Since practically all of the determinations were under this amount an accurate measurement was not attempted at the New York laboratory, but fortunately this was practically always done at the Vermont laboratory and the resulting measurements are given in the table.

A striking fact brought out by this table is the frequent failure to form gas which occurred in fermentation tests made with the same organism at different times. So marked was this tendency to vary that contradictory results were obtained with 21 of the 43 strains which were studied. This number would undoubtedly have been even larger if all of the strains had received an equal amount of study. Those with which the action on each of the sugars was tested on but two occasions make up 13 of the 22 strains among which variations were not noted. Taking the results as they stand there were 91 out of a total of 550 tests which did not show gas where it was found at other tests. Accordingly, when making a fermentation test in triplicate with one of these cultures the chances were over 16 per ct. that the conclusion reached was diametrically opposed to the truth, provided we accept the contention that a positive result is of more value than a negative result in fermentation test, proper care being used to prevent outside contamination and to provide uniform conditions. If this chance of error is considered as restricted to the strains with which variation was actually observed the above record does not express the actual gas-forming ability of the organism in 91 out of 347 observations, or 26 per ct.

The influence of the revivifying process on the certainty of a correct result is shown by comparing the error noted when this preliminary treatment was given with the error when it was omitted. Where the cultures had not been previously revivified, but inoculations were made into the fermentation tubes from young, actively-growing agar slopes, there was an error in 63 out of 189 observations, or 33 per ct. Where the cultures were given a preliminary cultivation as already ex-

plained (page 261), there were 28 errors out of 158 trials, or 18 per ct. This decrease of 15 per ct., or practically one-half, in the inaccuracy of the test brings out clearly the importance of revivifying cultures before attempting to determine their fermentative ability.

Again it will be noted that the failure to form gas during a part of the tests is not equally distributed among the three sugars, but that there are 47 cases with dextrose, 17 with lactose and 27 with saccharose. While it is true that a few more tests were made with dextrose than with either of the other sugars, this increase is not at all proportional to the larger number of failures.

In arranging Table III the organisms which had always formed gas from all of the sugars at all of the trials were placed first, followed by those in which failures to form gas had been noted. These latter were arranged in the order of the frequency of this failure down to Miller Stalk 3 No. 1 which, while it formed gas from each of the sugars during some of the tests, failed to do so in 13 out of 21 instances.

The fermentative vigor of these cultures was fairly proportional to this arrangement. The fairly distinct amounts of gas formed by the first cultures in the table gradually diminishes down the table until there is rarely more than a small bubble with dextrose in the case of CII, CIII, XCVIII, XCIV and Miller Stalk 3 Nos. 1, 2 and 3. The amounts of gas formed from saccharose also undergoes a like shading down, but its formation in appreciable quantities continues beyond the point where the formation of gas from dextrose ceases. Beginning with Potter's Bacillus there is a group which does not form visible gas from either dextrose or saccharose, but is able to form it from lactose. The remaining cultures in this table have never produced visible gas in the fermentation tubes in any of the determinations which have been made.

Riverhead Stalk 3 No. 1 is a significant member of the group connecting this last group with the one preceding it since in the earlier studies of this organism it fermented lactose with evident gas formation, but in all of the later

studies it failed to produce gas even after having been revivified. Although this germ apparently lost its ability to produce gas after the beginning of this study it should not be concluded that this was the rule, as it was really an exception. Owing to the stimulating effects of revivification which was practiced only during the latter part of the study a very considerable number of germs formed gas during the later tests when they had not done so during the earlier ones.

The fermentation tube has been largely used in the past to differentiate closely related species. It might be concluded in the light of the contradictory results which have been obtained in this study that the fermentation test is really of little value for this purpose. Such a general conclusion does not logically follow from this data because the particular group of organisms which have been studied here chance to lie on the very border line of visible gas formation and accordingly are not typical of germ life in general.

The fact that this group does lie on the border line of visible gas formation is shown by the fact that the amount of gas is small in most cases, in some cases only a small bubble appearing in the fermentation tube, and also by the failure of even this small amount of gas to appear in a considerable proportion of the tests with some of the strains.

The crudeness of the fermentation tube as a measure of the total gas formation is well brought out by Keyes.[24] He found in his study of *B. coli* that the total gas produced under comparable conditions in the fermentation tube and in a vacuum were in the ratio of 12.4 to 99.9.

In considering the importance which should be attached to the results obtained with the fermentation tube it should be remembered that all forms of living protoplasm respire gases and with the bacteria the carbon dioxide of respiration is an easily measurable quantity even with those forms which show no evidence of gas formation in the fermentation tube. The failure of the gas to appear in the tube is due to its solution in the liquid media and partial diffusion through the open arm

[24] Keyes, F. G. The gas production of *Bacillus coli*. *Jour. Med. Research*, N. S., 16: 69–82. 1909.

of the tube. The ability of the liquid to hold appreciable amount of gas in solution is illustrated by the bubbles of gas which appear in the top of the tube after the medium has been heated in the sterilizing process and again disappear on cooling.

While exact measurements are lacking it is probable that the gas of respiration does not nearly saturate the fluid in the fermentation tube so that the appearance of gas in the closed arm does not mark the beginning of the true fermentative action but rather a somewhat advanced stage of this action. Conversely, when dealing with a group of cultures which are on the turning point of the formation of visible gas, the failure of the gas to appear does not necessarily mean that the ability to ferment the sugar in question has been lost but rather that it has been so diminished as not to supersaturate the fluid in the tube. The fact of the continuation of the growth in the closed arm and of the formation of acid in these fermentation tube cultures lends color to the idea that the sugar is still being attacked in the same general way though less vigorously than before.

CLASSIFICATION.

Classification in a case like this may serve either of two distinct ends. It may assist future students to recognize the relationships of cultures which they may study and it may be of service to the farmer in showing whether the form which is present on a given crop will be dangerous to a succeeding crop which he may desire to grow later on the same soil. Because of the limited knowledge of germ life which is now available, any classification of such forms must be considered as tentative and as merely expressing the judgment of the authors concerning the relationships under discussion. In this connection it should be stated that some of the doubtful points in this classification were referred to six of our friends whose judgment was considered as of especial value in such matters. There was such a wide variation in what they considered the proper course to follow that it has seemed best to

confine the present publication largely to a presentation of the facts as observed and leave at least the more complicated portion of the classification until after the discussion of the pathology of these cultures.

It was brought out in Table II that these 43 cultures are practically identical with regard to the 38 headings under which they were there compared and that the only observed differences were those of fermentative ability which were clearly expressed by the group numbers.

It is seen from Table III that the first 15 cultures there given are identical in their fermentative ability in that all of them produce visible gas in the fermentation tube from dextrose, lactose and saccharose. If the principle is accepted that a single well demonstrated, positive result is conclusive the 18 additional cultures down to and including Miller Stalk 3 No. 1 must be held to be identical with the first 15.

Of this group of 33 cultures having the group number B. 221.1113022, *Bacillus carotovorus* Jones is the oldest described species and should be taken as the true representative of this collection of cultures. The two other cultures which have been described in literature as bacterial species, *Bacillus omnivorus* van Hall and *Bacillus olcreaceae* Harrison, are clearly identical with *Bacillus carotovorus* and there is no further occasion for continuing to recognize them as distinct species.

Continuing the arrangement of the cultures on the basis of the results from the fermentation tube the next group would have the group number B. 221.1123022 and is represented by the single strain Vermont XLVIII. This culture ferments dextrose and lactose with visible gas formation, but no apparent gas is formed from saccharose.

Mathematically the next group has the group number B. 221.1213022 and is likewise represented by a single strain, Vermont C. This group is characterized by the failure to form visible gas from lactose.

The next possibility is the failure to form gas from dextrose while forming it from the other two sugars under consideration and this group with a group number of B. 221.2113022

is represented by Vermont LIV and 0.2e. The faintness of the boundaries between these groups is shown by the case of 0.2e where in two determinations gas was actually formed in a single fermentation tube in each case. This result has not been accepted as conclusive because in neither case were the results from the accompanying tubes accordant.

The next group bears the group number B. 221.2123022 and is represented by Potter's Bacillus and Riverhead Stalk 3 No. 1. The former is a good illustration of the group while the latter forms a connecting link with the following group, since while it formed visible gas from lactose in the earlier tests it failed to do so during the latter studies and but for the earlier determinations would be included in the following group.

The series of groups is closed with a group of four cultures in which there was no visible gas formed from any of the sugars at any of the tests. This has a group number of B. 221.2223022 and *Bacillus aroideae* Townsend, is the only named species. Spieckermann's Bacillus was isolated and described at an earlier date, but was not given a specific name.

Summarizing this arrangement on the basis of the results from the fermentation tube test with dextrose, lactose and saccharose we have the following:

B. 221.1113022 *Bacillus carotovorus* Jones and 32 other cultures.
B. 221.1123022 Vermont XLVIII.
B. 221.1213022 Vermont C.
B. 221.2113022 Vermont LIV and 1 other culture.
B. 221.2123022 Potter's Bacillus and 1 other culture.
B. 221.2223022 *Bacillus aroideae* and 3 other cultures.

It will be observed that the two possible groups of .221 and .122 are not represented. It is really surprising that in a collection of 43 cultures there should have been examples of six different groups and there is no apparent reason why a study of a larger number of cultures should not bring out these missing ones.

The above arrangement is one which will appeal to the students of this field because it clearly summarizes the observed results and provides a type for all of the cultures which will be found except those which belong to the missing groups above referred to. Unfortunately this clearness of classification is more apparent than real, since practically each successive determination of the collection of cultures led to a rearrangement of the representatives of the various groups with a gradual shifting toward the upper groups because of the greater importance placed on a positive result than on a negative one. The final accumulation of 33 cultures in the upper group is largely the expression of the continued action of the law of chance and had the study continued longer this group would undoubtedly have been correspondingly enlarged.

An inspection of Table III shows that while some of the organism gave constant results at the various determinations a considerable number vibrated from one end of the above set of group numbers to the other at different determinations, often being classed temporarily with a number of the intermediate groups. Under such circumstances the above classification is seen to represent divisions which are too shifting and transient to be designated as species.

While the authors do not desire to be dogmatic in this connection the conception has been forced upon them during this study extending over a series of years that they were dealing with a group of organisms which were very closely related and which combined a remarkable stability and uniformity with regard to practically all of their culture characteristics with a remarkable variability with regard to the results from the fermentation tube test with certain sugars. It would seem that the correct explanation of this apparent variability was the fact that the entire group had a very weak fermentative ability and the gas formed from the sugars in question was approximately equal to the amount required to saturate the fluid in the fermentation tube and provide for the diffusion which is unavoidable in that test. With the changes

in the fermentative vigor of the culture, concerning the details of which comparatively little is known, the amount of gas produced fluctuates above and below this saturation point giving the accompanying positive and negative results based on the presence or absence of visible gas in the closed arm of the fermentation tube.

The important fact of this fluctuation having been recognized it is a matter of little consequence to the scientist whether it is maintained that there are six species or only one. It should be mentioned in passing that the idea of species as originally conceived is entirely inapplicable to bacteria since it was based on morphological similarity coupled with an ability to produce fertile offspring. Morphologically the entire bacterial world can be divided into only a few groups and sexual reproduction is entirely lacking. In the present instance it seems to the authors that the only possible basis for recognizing more than one so-called species in connection with the present group of cultures must lie in the relation of the parasite to the host and since that part of the subject will be treated in a succeeding publication the matter of classification will be dropped at this point.

DETAILED DESCRIPTION.

On the Classification Card of the American Society of Bacteriologists in addition to the group number and the material given in Table II, there is a provision for the detailed characteristics of the organism in question. It was the original intention to present this description for the typical representative of each of the groups of cultures as given above, but when these descriptions had been prepared it was found that they were practically verbatim copies with the exception of the fermentation of sugar which has already been discussed. Accordingly the description of *Bacillus carotovorus*, Jones will be given and with the exceptions just noted this may be accepted as applying to the entire collection of cultures.

Bacillus carotovorus Jones.

For group number and brief characterization see page 264.

I. MORPHOLOGY.

1. Vegetative cells. Medium used, agar slope at 20-25 C. 1-2 days old; Form, *short rods, long rods, short chains, long chains;* Limits of size, .7 to 1 x 1.5 to 5; Size of majority, .8 by 2; Ends, *rounded.*
3. Endospores, *none.*
4. Flagella, 2 to 10; Attachment, *peritrichiate;* How stained, *Pitfield, Löwits, Fischer.*
5. Capsules, *none.*
6. Pseudozoogloea, *present, slight.*
7. Involution forms, *not observed.*
8. Staining reactions. Stains well in *watery fuchsin, gentian violet, carbol fuchsin, Loeffler's alkaline methylene blue;* Gram, *negative.*

II. CULTURAL FEATURES.

1. Agar slope. Growth, *abundant;* Form of growth, *filiform* to *spreading;* Elevation of growth, *effuse* to *raised;* Luster, *glistening;* Typography, *smooth* to *contoured;* Optical characters, *opaque* to *opalescent.*
2. Potato. Growth, *moderate* to *abundant;* Form of growth, *filiform* to *spreading;* Elevation, *effuse* to *raised;* Lustre, *glistening;* Typography, *smooth* to *contoured;* Chromogenesis, *white on all media;* Odor, *decided;* Consistency, *butyrous;* Medium, *grayed,* but *not discolored* in the customary meaning of that term.
4. Agar stab. Growth, *best at top, abundant, wide-spreading;* Line of puncture, *filiform.*
5. Gelatin stab. Growth, *best at top;* Line of puncture, *filiform;* Liquefaction, *crateriform* to *infundibuliform;* Begins on *2d day* at 20° C.; Complete in *6 days* with some cultures and *not in months* with others.

6. Nutrient broth. Surface growth, *pellicle thin* and *sometimes absent;* Clouding, *moderate* to *strong, persistent;* Odor, *decided;* Sediment, *compact* to *flocculent, usually abundant.*
7. Milk. Coagulation, *usually on 3d day* at 20° C.; Coagulum, *very slowly and slightly peptonized,* and not complete *in months,* digestion *not clearly evident to the eye;* Medium, slightly *browned.*
8. Litmus milk. *Acid, litmus reduced.*
9. Gelatin colonies. Growth, *rapid;* Form, *punctiform* to *round;* Depression, *crateriform;* Edge, *entire;* Liquefaction, *saucer.*
10. Agar colonies. Growth, *rapid* at 20-25° C.; Form, *round, occasionally irregular,* deep colonies *fusiform;* Surface, *smooth;* Elevation, *raised* to *convex; Edge, entire* to *undulate;* internal structure, *amorphus* to *coarsely granular* or even *grumose.*
13. Cohn's solution, *no growth.*
14. Uschinsky's solution, *abundant.*
18. Best medium for long continued growth is peptone bouillon.
19. Quick test for differential purposes. Slices of uncooked carrots, turnips and cabbages.

III. PHYSICAL AND BIOCHEMICAL FEATURES.

1. Fermentation tubes. Gas[25] produced in small amounts from *dextrose, lactose* and *saccharose,* but not from *glycerin;* Growth in the closed arm with *dextrose, lactose* and *saccharose,* but not with *glycerin;* Acid produced from *dextrose, lactose, saccharose* and *glycerin.*
3. Nitrates in nitrate broth *reduced* to *nitrites.*
4. Indol production *feeble.*
7. Optimum reaction for growth in bouillon in terms of Fuller's scale, 0.
8. Vitality on culture media, *moderate.*

[25] See page 271.

9. Temperature relations. Thermal death point, 48-50° C.; Optimum, 25-30° C.; Maximum, above 38° C.; Minimum, below 10° C.
10. Killed *readily* by drying.
12. Sunlight. Exposure, not on ice, at midday in Sept. showed decrease after 1 minute, increasing to destruction of 90-100 per ct. after 20 minutes.

IV. Pathogenic to many fleshy vegetables of the North Temperate Zone.

CONCLUSIONS.

A considerable number of the cultivated plants in the North temperate zone suffer at times from a bacterial soft rot caused by a non-chromogenic, liquefying bacillus.

This comparative study of forty-three pathogenic strains derived from six different vegetables indicates that the results of fermentation tube tests with dextrose, lactose and saccharose offer the only usable cultural basis for differentiating these strains. However the cultures of the entire group have a weak fermentative power which, with very few exceptions, produces only a little more than enough gas to become evident in the fermentation tube. At other times the same strains produce no gas at all. These variations make the results of the fermentation tube test an unsatisfactory basis for classification.

Unless later studies of the pathogenicity of these cultures shall offer a basis for subdividing them, there is no apparent reason why they should not all be considered as somewhat variant members of a single botanical species.

II. PECTINASE, THE CYTOLYTIC ENZYM PRODUCED BY BACILLUS CAROTOVORUS AND CERTAIN OTHER SOFT-ROT ORGANISMS.

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II. PECTINASE, THE CYTOLYTIC ENZYM PRODUCED BY BACILLUS CAROTOVORUS AND CERTAIN OTHER SOFT-ROT ORGANISMS.[1]

L. R. JONES.

INTRODUCTION.

Two of our earlier publications (1900, 1901) have given detailed accounts of a bacterial soft rot of carrot and other vegetables due to the organism, *Bacillus carotovorus*. In the first of these articles it is stated (1901: 304, 312)[2] that "microscopic examination of the decaying carrot tissues has shown that the organism invades the intercellular spaces, and multiplies there with enormous rapidity. The middle lamellae of the adjacent cells appear to be softened or destroyed by the secretions of the bacteria, since isolation of the cells in invaded tissue occurs, but the bacteria have not been observed in the interior of the cells of the recently disorganized tissues. ..... This (action on the middle lamella) is probably due to an enzym of the nature of cytase excreted by the bacteria. ..... It seems probable that the further study of the bacteria concerned with the soft rots of plants will swell the list to a considerable number of organisms which resemble the above and each other more or less closely in their physiological characters."

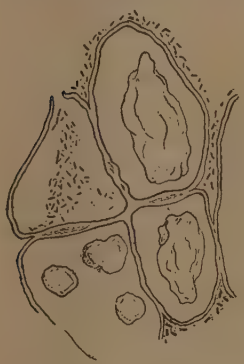

FIG. 1. CARROT-ROOT PARENCHYMA IN EARLY STAGE OF DECAY CAUSED BY *B. carotovorus*.

The cytoplasm is plasmolyzed and the bacteria confined to the intercellulars except where the wall has suffered mechanical rupture, as in the upper left-hand cell.

It appears, therefore, that a fuller understanding of the cell-wall-dissolving or other enzyms produced by such bacteria is desirable for two practical reasons: First, because the parasitism in each case seems directly associated

[1] The laboratory studies upon the enzym here discussed were made during the years 1901–04, and this manuscript was prepared at their completion in 1904. Owing to the plan of cooperation, as explained in Part I, this pub-

with and probably dependent upon the power to produce such an enzym. Second, because these soft rot organisms are so much alike as to make the question of their specific relationship an extremely complex one which must ultimately be settled largely by appeal to physiological characters, including their ability in parasitism, and it would seem that their enzym production might prove of value in this connection as a differential character. Another interesting question of fundamental importance concerns the relationship of the cytolytic enzyms, or cytases, from various sources. We have attempted to determine whether the enzym here dealt with is identical with those obtained by de Bary (1886)[2] and Ward (1888) from certain fungi, by Brown and Morris (1890) and later investigators from seeds and by Potter (1899) and others from soft rot bacteria.

In the progress of these investigations a detailed study was first made of the enzym as produced by the carrot-rot organism, including a comparison of its action when secured in various ways apart from the living bacillus, and also as observed upon the sundry vegetable tissues. Thereafter a comparison was undertaken of the characteristics of the enzyms secured from the soft-rot organisms of several other vegetables and finally with wall-dissolving enzyms produced by other classes of bacteria, by fungi and by germinating seeds. In the following discussion of the results it will conduce to clearness and directness of statement to follow a somewhat similar order. It might seem more logical to begin with a

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lication has awaited the conclusion of the associated morphological studies, except for a summarized account of the work which appeared in the "Centralblatt für Bakteriologie und Parasitenkunde," 1905, (see Bibliography). Inasmuch as no important contribution to the subject has come to our attention meanwhile, it has seemed best to leave the manuscript unaltered, and to add no titles to the bibliography later than 1905.

An important part of the work of the author was done in the botanical laboratory of the University of Michigan where he profited from the advice of Professor F. C. Newcombe. In the investigations carried on in his own laboratory in Vermont, valuable assistance was had from two of his students, Messrs. H. D. Bone and L. P. Sprague. He gratefully acknowledges his indebtedness to these gentlemen.

[2] All citations to the bibliography follow the plan of giving in parenthesis after the author's name the year of publication, followed where desired with the page numbers specifically referred to.

detailed discussion of the action of the enzym upon cell walls. In pursuance of the above plan, this is deferred and it must here suffice to state that the action consists in the softening and swelling of the walls and ultimately in the complete solution of their middle lamellae in susceptible vegetable tissues, but that in all cases it stops short of complete solution of the cell membrane, a residue of cellulose always remaining.

The studies here reported upon were carried on at various times during the years 1901-1904. The strain of the carrot-rot organism used has been the same throughout and is that isolated from decaying carrot tissues in 1899 and since carried in culture, practically all of the time in beef broth, at the ordinary laboratory temperature, 16°-22° C.

The question has arisen as to whether during this time reduction has occurred in pathogenicity and rate of enzym production. Van Hall (1903) found that his iris rot organisms lost pathogenicity to iris after only four weeks in laboratory culture and his experience with these led him to expect this as a rule with such organisms. Laurent's (1899) and Lepoutres' (1902) experiments suggest a like probability. Potter (1900: 445) found no such rapid loss of pathogenicity in his turnip rot organism.

In the case of *B. carotovorus* there has, in our judgment, been a considerable decrease in pathogenicity since our first trials of 1899. The change has, however, been a gradual one rather than rapid or radical, and, therefore, difficult to estimate. The virulence and rate of invasion of tissues has always varied with vegetables and environmental conditions, but it is certainly less vigorous in attacking even the more susceptible plants now than it was in our earlier inoculation experiments. A culture of this organism was sent to Messrs. Harding and Stewart of the New York Experiment Station in 1902. They reported it actively pathogenic at first, 1902, but wrote us a year later that it had lost practically all pathogenicity. Meanwhile our own cultures had not changed in any such radical way. In this connection it is worthy of remark that Harding and Stewart kept their culture on agar whereas ours were carried in broth. *B. carotovorus* is

remarkably sensitive to desiccation as shown in our earlier work (1900: 328). It seems to us not unlikely that the weakening observed at the New York Station may have resulted from the intermittent desiccation incident to its culture on agar.

Exact estimates as to relative ability in enzym production shown now and formerly are even more difficult to make than are those as to pathogenicity. Our first isolations of enzym were made in 1901. We have compared some of the preserved samples of these earlier enzyms with those isolated in 1903. Those of 1901 were certainly more active than those of 1903. This difference might, however, possibly be accounted for by variations in medium and vigor of development as shown later in this article.

In our judgment, along with the loss of pathogenicity there has been a corresponding decline in enzym-producing power, but both of these have been so gradual as to be hard to estimate quantitatively.

The first thing undertaken in the course of these investigations was to determine whether the softening of the tissues associated with the invasion of this bacillus was certainly due to an enzym, and whether, if so, the enzym was separable from the organism. Five methods were tried with the object of securing such enzym, if it existed, apart from the organism, viz., (1) heat, (2) filtration, (3) germicides, (4) diffusion through agar, (5) precipitation by alcohol.

The first three methods involved, in all cases alike, the following procedures: The cultivation of the organism in beef broth for periods varying from three to fourteen days; the treatment of such cultures by the methods under trial; the determination of the sterility in the broth so treated; in case sterility was secured, the testing of the cytolytic activity of this sterilized broth by immersion in it of sterile blocks cut from fresh uncooked carrot or turnip roots, or from potato tubers or of cotyledon of immature pea; finally, tests to determine the continued sterility of the broth during this last trial period. In all cases where chemicals were used control trials were made to be sure that the chemical itself was not the cause of the changes observed.

STUDIES WHEREIN THE ENZYM WAS ISOLATED BY HEATING.

The thermal death point of the carrot-rot bacillus was carefully determined in connection with our earlier studies. It was found that recently inoculated thin-walled-tube cultures immersed for ten minutes at 51° C., or slightly under this, are rendered sterile.[3]

Previous studies (cf. Green, 1901: 98) upon cytolytic enzyms have shown that when in solution they are destroyed by heating to a temperature of 60°-65° C. It seemed probable, assuming that we are dealing with the same or a similar enzym, that there might be an intermediate temperature where the organism would be destroyed and the enzym left in the solution. This matter was tested by cultivating the organism in beef broth, heating these cultures to sterilize, making transfers to prove sterility and inserting bits of sterile carrot or other fresh vegetable tissues to determine enzym action.

The details of a single experiment will suffice to make clear the methods and aid in interpreting the results.

A series of six 10 c. c. beef broth cultures seven days old were immersed for ten minutes in a water bath of which the temperature was held at 55° C. These tubes were of thin glass about 15 x 1.5 cm. in size, immersed in the water three-fourths of their depth. The original reaction of the broth was +1.5%. To make sure of continued sterility transfers were made from the tubes soon after leaving the bath and again at the close of the experiment. Immediately after heating, a small block of living carrot tissue, cut from the interior of the root with proper precautions to insure sterility, was inserted into each tube. Like blocks of tissue were put into control tubes of each of two kinds, first, sterile uninoculated broth, and second, living broth cultures of the same age as the ones heated. The result was that the tissues in sterile uninoculated broth remained unsoftened, those in the living cultures rapidly softened and were fully decomposed in three

[3] The above trials were first made in 1899 and repeated with like results in 1901. A careful repetition in 1903, using the same methods, showed a thermal death point fully one degree lower. This is doubtless the result of long cultivation in the laboratory. It is simply a matter of biological interest, which does not in any way affect the methods or results of the enzym studies discussed above.

or four days, while those in the heated tubes showed a similar but slower softening, requiring ten days for full action.

In other experiments sterile blocks cut from turnip root and cotyledons of immature peas were used and were similarly softened. The results were fairly uniform and satisfactory. Various cultures so exposed at temperatures of 54°, 55°, 58°, 60°, 62°, 63°, 64°, 65°, 68°, 73° were rendered sterile in practically all cases. There was distinct cytolytic action in the sterile broths heated at the lower temperatures and none in those heated at the higher. There was, however, evident inhibition of activity even at the lowest of these as is shown in the experiment just described. Heating at 60°-62° inhibited the action to a marked degree as compared with 58° and in all cases, except one, heating at 62° entirely checked the activity. In only one case did any action occur in tubes heated at 63° and that was probably explainable on the ground of erroneous reading of temperature, since a repetition of the work gave results in harmony with the other series. No action occurred in any tube heated above 63°. The point of total inhibition of the cytolytic action as determined by this method, therefore, lay at or about 62° and there was marked decrease at all temperatures above 58°. Certain temperature relations will further be discussed later.

STUDIES WHEREIN THE ENZYM WAS SECURED BY FILTRATION.

It seemed probable that if the enzym were in solution outside the bodies of the bacilli it would pass through the porcelain bacterial filters and so be obtained apart from the organisms. Broth cultures of ages varying from seven to fourteen days growth have on six different occasions and with different bougies been passed through the Pasteur-Chamberland filters and the sterility and enzym content of the filtrate tested. There has been no difficulty in securing sterility with Pasteur-Chamberland filters, although earlier attempts with thinner walled and probably less perfect bougies were not successful.

Numerous trials were made by immersing in this sterile filtrate sterile cubical blocks cut from fresh roots of carrot and turnip and from potato tubers and also cotyledons of young peas, either fresh or those which had been for some time in sterile broth. In all cases alike these tissues were softened with solution of the middle lamella as in the presence of the living organisms. The detailed record of a single experiment will suffice.

In each of six tubes containing 10 c. c. of the sterile filtrate was introduced a carrot block about 3 mm. in diameter. At the end of twenty-four hours there was perceptible softening over the surface of these blocks and at the end of three days they were softened throughout. Potato blocks of similar size tested in the same way showed the first signs of softening on the fifth day and required ten days for complete softening.

These experiments and other similar ones that might be cited, including trials with razor sections under the microscope, showed that the lamella-dissolving enzym was in solution in the broth outside of the bodies of the bacilli. The question arose, however, as to whether the filtrate possessed the full enzymic activity of the original broth. It is conceivable that the bodies of the bacilli contain much of the enzym which gradually diffuses into the external liquid even after their death, and also conceivable that the filter may retain some of the enzym which was diffused in the original broth. To gain information on these points the enzymic activity of the filtered sterile broth was compared at various times with unfiltered broth cultures and with cultures of broths sterilized by the addition of chemicals. In one series of trials thin razor sections from roots of each carrot and turnip were immersed in (a) culture broth sterilized by filtration, (b) culture broth sterilized by a 20% addition of chloroform, (c) solutions of alcoholic precipitate from culture broth. The solutions (b) and (c) acted about alike, whereas (a) required at least twice as long to disintegrate the tissues. Similar trials made later using razor sections of turnip showed no difference of importance between the cultures containing the living organism

and sterile broths like (b) and solutions like (c). A detailed discussion of these points occurs later.

Similarly sterile blocks from living carrot root were placed in the filtrate in comparison with like blocks in living cultures and others in broth plus 10% of chloroform to sterilize. The latter were shaken thoroughly and sterility proved. The carrot tissues in the living cultures and in the tubes sterilized by chloroform were alike softened in three days, whereas the blocks in the filtrate were more slowly acted upon, requiring nine days for full action. Still further evidence of this relation of filtration to enzym content was obtained by the method of alcoholic precipitation to be discussed later. Samples of filtered and unfiltered cultures were rendered 80% alcoholic and the filtered yielded only one-fourth as much of the enzym-containing precipitate as did the unfiltered. Moreover five per ct. solutions of each of these tested upon razor sections of carrot and turnip showed the enzymic action of the unfiltered fully twice as rapid as that of the filtered. Taking into consideration both the relative amounts and the relative strengths of these solutions, it would seem from this last trial that possibly four-fifths of the enzym was lost by filtration through the porcelain.

Comparisons and conclusions.—All these experiments give like evidence that passage through the Pasteur-Chamberland filters as used in these trials reduces decidedly the enzym content of the broth, although it does not remove all of it. Just why this retention of the enzym occurs has not been determined. As already suggested, this may be in part, at least, the enzym contained within the bacterial cells and which would later diffuse into the surrounding liquid; or it may be in part or wholly external to these cells either closely associated with the bodies of the bacteria and so retained with the bacterial slime in the porcelain; or it may be that the filter removes some of the enzym content which is diffused or in solution in the broth. The results of Freudenreich (1899) point to the latter conclusion. He attempted to clarify cheese extract for qualitative analysis by passing through Chamberland fil-

ters. He found that although the crystallizable and diffusible nitrogenous compounds (amids) passed through the bougies, some 90% of the soluble protein matter might be held back. Although all the bougies used by him retained considerable of the nitrogenous matter his trials showed that this passed much more fully through a new one than through the same after it had been used several times. In later trials (1900) he passed milk through these filters and found that the enzym galactase was removed thereby.

These experiments of Freudenreich came to our attention after we had completed our filtration experiments. In reviewing our records in their light we find that we used new filters or those which had been used for similar work only a few times. We are assured, therefore, that even the new bougies largely reduced the enzym content and that none that we employed, some of which had been used several times, wholly eliminated it.

The results of others who have tested the relation of filtration to enzym content of cultures of similar soft-rot bacteria may profitably be reviewed in this connection.

Potter (1900: 448) found that filtration through Pasteur-Chamberland filter did not remove the enzym produced by his *Pseudomonas destructans.* Laurent (1899) found similar bacterial enzyms to pass through porcelain while Spieckermann (1902) found that after passing culture broths through the Reichel porcelain filter the sterile broth had not the least enzymic action. Van Hall (1902) found that the juice expressed from potato decayed by the invasion of *B. subtilis* when passed through the porcelain filter retained the property of rapidly destroying the potato tissue. He also found (*Zeitschr. f. Pflkr.,* 1903) similar but weakened action in the juice from iris invaded by his *Bacillus omnivorus,* when this was passed through a porcelain filter. In other trials he found filtered broths lost all activity. We are at a loss to reconcile some of these results with our own and the others except by appealing to differences in the filters.

STUDIES WHEREIN THE ENZYM WAS ISOLATED BY THE USE OF GERMICIDES.

The preceding results were satisfactory so far as they went, but it was manifestly desirable to find some simpler method of procedure. Two serious objections to the preceding methods should be eliminated if possible, namely, first, that precautions are necessary to insure the sterility of the broths during prolonged study subsequent to filtration; and, second, the ever-present danger that the method used to eliminate the organism may at the same time remove or weaken the enzym. It seemed probable from the experience of others who have studied similar enzyms that some chemical added to the cultures would kill or wholly inhibit the bacillus without destroying the enzym or interfering with its activity. With this hope, trial was made of additions of formalin, phenol, thymol, and chloroform, respectively, to beef broth cultures. In considering the results reference was also made to related experiments, to be discussed in detail later, where similar additions of these chemicals have been made to solutions of the enzym-containing alcoholic precipitates.

FORMALIN.

The relation of formalin, both to the life of the organism and to the activity of the precipitated enzym, has been determined. It has been found that both the organism and the enzym are extremely sensitive to this chemical. Since, however, the organism is more so it is possible so to gage the amount as to sterilize the broth and leave the enzym active. These conclusions are based upon experiments made by the addition of varying proportions of formalin, both to the beef broth cultures and to solutions of the precipitated enzym. Some hundreds of such additions have been made to beef broth cultures, including the following strengths, and numerous trials each of many of them: Formalin 0.03%, 0.06%, 0.08%, 0.095%, 0.18%, 0.33%, 0.46%, 0.57%.

The detailed account of a single series will suffice to explain the general method and results. All this work was carried on at temperatures of 18°-22° C.

To ten broth tube cultures, 10 c. c. each, formalin was added as follows and the tubes then thoroughly shaken: Two tubes (1 & 1′), 0.57% formalin; two tubes (2 & 2′), 0.46%; two tubes (3 & 3′), 0.33%; two tubes (4 & 4′), 0.18%; two tubes (5 & 5′), 0.095%. Two tubes (6 & 6′) containing sterile broth were included to serve as controls. On the third day transfers made from these to sterile broth proved the sterility of all. On the fifth day a sterile cube 5 mm. in diameter, cut from the interior of fresh carrot was inserted into each tube. On the eleventh day the carrot tissues in tubes 1, 1′, 2, 2′, 3, 3′, 6, 6′, were not softened; those in 4, 4′, 5, 5′, were completely softened. On the fourteenth day 3, 3′, were softened somewhat; others unchanged. On the twenty-third day 3, 3′, further softened; 2, 2′, softened somewhat but less than 3, 3′. On the thirty-third day 1, 1′, and 6, 6′, showed no softening; others completely softened. Final transfers to sterile broth showed all tubes sterile at the close of the trial.

As a result of similar series, repeating the above and using other strengths of formalin, the following conclusions have been reached. The addition of 0.1% formalin is sufficient to sterilize a beef broth culture of *B. carotovorus* one to ten days old, providing the tube is thoroughly shaken. More formalin, 0.2% or even more, may be needed if not thoroughly shaken. The presence of 0.6% or more formalin completely inhibits enzym action; amounts as low as 0.3% retard to a marked degree. There was perceptible retardation from 0.06% formalin, although this amount was too slight to sterilize with certainty. In all the above trials the formalin acted on the broth several days before its relation to the enzymic activity was determined.

Trials were also made to determine the effect of formalin additions to solutions of the enzym obtained from broth cultures by precipitation with alcohol. The activity in these cases was determined by trial on razor sections of carrot roots. The results were in accord with those just discussed, viz., a slight but appreciable retardation from 0.05% addition, and almost complete inhibition where 0.5% was added. In these latter trials the formalin was added to the enzym solution some time before the cytolytic activity of the mixture was

determined. The results led us to abandon further work with formalin.

After the preceding work with formalin was completed, however, Spieckermann's article (1902: 166) reached our hands in which he reports that a 0.2% solution of formalin sterilized the cultures of the soft-rot organism of cabbage with which he was working and did not inhibit the action of the cytolytic enzym, *at least for several hours.* We, therefore, undertook to learn the relative rate of action of formalin upon each, *B. carotovorus* and its enzym, by adding to broth cultures 0.2% of formalin, shaking thoroughly and testing both viability and enzym action at frequent intervals. It was found in the first series of trials that at the end of twenty-four hours the activity of the enzym was not appreciably lessened; at the end of forty-eight hours there was slight retardation and this was pronounced at the end of seventy-two hours. On the other hand transfers at the end of only three hours showed most of the organisms to be dead or so affected that growth was slow in starting and at the end of forty-eight hours the broth was sterile.

In a second series of trials the broths were tested at more frequent intervals. This again showed the organisms to be killed before the enzym was fully destroyed, but the retardation was more pronounced than in the preceding trials. The details are as follows:

1. Action on enzym:—Broth cultures six days old; formalin added to make 0.2% solution, thoroughly shaken; cytolytic action compared with control tubes by testing upon thin sections of turnip. Result: After three hours slight retardation was evident; after six hours nearly twice as long a time was required for the same results in the formalin solution, i. e., formalin retarded the action 50%; after twenty-four hours still more difference; after forty-eight hours formalin solution required five times as long as control; after eighteen days it required sixty hours to disorganize sections, whereas the control broth did this in one hour.

In a third series, little if any retardation up to the sixth hour; after nine hours the action of the formalin broth re-

quired twice as long; after twenty-four hours it required four times as long.

2. Action on the organism:—Formalin to make 0.2% solution was added to broth cultures, thoroughly shaken; transfers at the end of each two, three, six, and nine hours showed growth. There was, however, progressive retardation. Thus the control was clouded in less than twenty-four hours; that made at the end of two hours showed clouding first on the third day; that at the end of three hours first clouded on the fifth day; six hours, on the seventh day; nine hours, on the twelfth day. After clouding appeared growth progressed with normal rapidity.

These results show sufficient variation between the different trials to forbid sweeping generalizations. They agree, however, with each other and with Spieckermann's results in showing that the action on the organism is more rapid than on the enzym. There was no appreciable retardation of the enzym action until after a period varying from three to nine hours, or in one case twenty-four hours, whereas there was marked inhibition in growth of the organisms after two or three hours. These results were of such a nature, however, as to discourage us from looking to the use of formalin as a practical method of sterilizing broths preparatory to the study of the normal action of the enzym. If used within two or three hours as Spieckermann directs, sterility is not insured; if a longer time elapses, the activity of the enzym will be reduced quantitatively at least and conceivably affected qualitatively.

Bliss and Novy (1899: 52) have shown that fibrin, which has been acted upon for a short time by formalin, resists thereafter the digestive action of proteolytic enzyms. These observations raised the question as to whether the retardation in the cytolytic action already noted might be in any degree the result of the action of the formalin on the wall of the vegetable tissues rather than upon the enzym itself. In order to determine this, razor sections of turnip and radish were immersed twenty-four hours in full strength formalin, then washed out in water, and the rapidity of action of enzym solution on these compared with that on freshly cut sections

and on those which had lain twenty-four hours in absolute alcohol. Other similar trials were made where the tissues had lain a month in either formalin or in absolute alcohol, respectively. All were promptly and similarly decomposed, there being no evidence of difference in this respect. The inhibiting action of formalin must, therefore, be attributed to its effect on the enzym itself rather than on the tissues. It is interesting to note in this connection that Bliss and Novy (1899: 79) found formalin to inhibit certain enzyms (papain, trypsin, amylopsin) and not others (pepsin, malt diastase). Von Freudenreich (1900), experimenting upon milk enzyms, found that formalin tends to lessen the action of galactase more promptly than it does that of pepsin and pancreatin.

It is surprising that Potter (1900: 448) was apparently unable to destroy with formalin the organism causing white rot of turnip. We are led from our experiments to believe that larger amounts of formalin or more thorough agitation would have accomplished this.

PHENOL.

Seven trials of this, each involving several cultures, were made with uniformly satisfactory results. A piece of the crystal varying in size from one-fourth to one-half that of a pea, (i. e., making 0.3% to 0.6% solution) added to a 10 c. c. broth culture and well shaken has never failed to produce sterility and there is apparently no retardation of the activity of the enzym. To cite a single experiment:

A crystal of phenol half size of a pea, i. e., making about 0.5% solution was added to each of six 10 c. c. broth cultures six days old and thoroughly shaken. On the second day thereafter transfers were made to test sterility. No growth having developed from these transfers seven days later, sterility was inferred and a cube of sterile fresh carrot about 5 mm. in diameter was added to each tube. In two days' time all these pieces of carrots were fully softened. When compared with other tubes sterilized by thymol or chloroform and with those containing the living organism such phenol tubes showed no evidence of retardation.

In other trials it was found that additions of 0.1% and less of phenol failed to sterilize and, on the other hand, additions of 5% or more totally inhibited the activity of the enzym. The phenol was added to the culture in these experiments four days before the enzymic activity was tested.

THYMOL.

This proved less satisfactory as a germicide than phenol, but chiefly, we think, because of its slight solubility and slow diffusion in the broth. If the alcoholic solution is used the thymol is precipitated upon contact with water, hence there is no gain. Our trials have shown that powdered thymol, which floats on the surface of a broth culture, will sterilize the surface layer, but that where the culture has stood without shaking, living organisms persist, at least for many days, in the deeper parts of the broth. For this reason even large amounts of thymol will fail to sterilize in the absence of agitation, whereas sterility can be secured with small amounts by thorough shaking. The following experiments will serve to show this.

A large excess of thymol (2%) was added to each of ten broth tube cultures eight days old and a small amount (about 0.2%) to each of ten similar tubes. All of these were left without agitation. Transfers made on the eighth day thereafter showed living organisms in four of the tubes containing the larger amount of thymol and in five of those with the smaller amount. On the tenth day living organisms remained in three tubes of each series. In another series a crystal of thymol equalling about 0.2% of the broth was added to each of three ten c. c. broth tube cultures four days old and a tiny crystal of about one-fourth this size to each of three other similar cultures. These were all thoroughly shaken. Transfers from these tubes on the second day thereafter showed living organisms in all three of these containing the lesser amount, but the others were sterile. In all cases pieces of sterile carrot inserted into tubes sterilized by thymol have been quickly softened without evidence of inhibition.

In comparison with the above results it is of interest to note that Potter failed to sterilize cultures of his turnip white-rot organism by the use of thymol (1900: 448); but the possibility remains that more agitation would have secured sterility in his cultures.

E. F. Smith (1901) found that certain organisms will grow in beef broth in the presence of thymol, but his statements would indicate that there was little or no agitation of the broth. These results force us to question whether full dependence can be placed on some of the results of Bourquelot and Herissey's work on pectin enzyms, as discussed later in this article, since they, apparently, depended on additions of thymol water to insure sterility.

CHLOROFORM.

Since this is the agent usually employed in enzym studies for the inhibition of bacterial growth especial attention has been given to the determination of its relation both to the organism and the enzym.

The first experiments to determine this were carried out in 1901. In these Powers & Weightmann chloroform of "U. S. P. standard" strength was used. This was added to broth tube cultures, seven to nine days old, in amounts to give proportions varying in different experiments from 10% to 50%. These tubes were shaken, then allowed to stand and stratify. The excess of chloroform promptly settled to the bottom, but such tubes continued to emit a strong odor of chloroform throughout the experiments. Transfers made three days later showed the cultures to contain living organisms in all cases.

During the year 1903, these trials were repeated, using both Mallinckbrodt's "M. C. W. purified" chloroform and the "U. S. P." grades both of this firm and of Powers and Weightman. These later results were alike in all trials and differed from those made in 1901. In every one of these later cases where 10% or more of chloroform was used and *the tubes thoroughly shaken* sterility was secured.

The details of a single trial will suffice to illustrate the method and results. Chloroform was added as follows to each of six 10 c. c. broth tube cultures, six days old, shaking very thoroughly; to tubes 1 & 1′ added 1 c. c. of chloroform; to tubes 2 & 2′ added 0.5 c. c. of chloroform; to tubes 3 & 3′ added 0.3 c. c. of chloroform. On the third day thereafter transfers from these showed 1 & 1′ to be sterile, whereas the others contained living organisms. On the fifth day transfers from these latter again showed living organisms. Cubical blocks of fresh carrot were placed in tubes thus sterilized with 10% additions of chloroform and in other similar series sterilized by 25% and 50% additions respectively, and in all cases alike they were quickly and fully softened.

There was no appreciable retardation in the rate of softening in any such case as compared with tubes sterilized with thymol or phenol, or even with cultures containing the living organisms.

This matter was of so much importance that further comparative trials were made by using razor sections of turnip. There was no appreciable difference in the rate of softening as between living cultures and those sterilized by chloroform. Thus in one trial, broth cultures four days old were used; 10% of chloroform was added to each of two of these; after thorough shaking immediate trials were made comparing the activity with that of similar cultures; no difference was found. Again, at the end of the sixth day when the chloroform tubes were sterile, comparative trials showed the sterile broth to equal in enzymic activity the living control cultures, now ten days old.

Comparisons and final conclusions.—These results showed chloroform to have no inhibiting effect upon the enzym when used even in great excess and proved the efficiency of chloroform as a germicidal agent in such work as we were doing. At the same time they emphasize the need of painstaking and caution if chloroform is relied upon, either to sterilize cultures or to preserve sterility of enzym solutions as is so frequently done.

A comparison of these results with those obtained by others will again prove helpful.

Brown and Escombe (1898: 16) satisfied themselves that the cytolytic enzym of barley is not appreciably weakened in its action by a saturated aqueous solution of chloroform. Smith (1901) has called attention to the fact that many organisms are surprisingly resistant to chloroform and emphasized the point we have just made, as to the need of caution in its use. Potter (1900: 448) did not succeed in sterilizing cultures of the turnip white-rot organism with chloroform, but Spieckermann (1902: 166) found chloroform effective for sterilizing the sap of vegetables invaded by his kale rot organism. He does not state the amount used nor the method of agitation, but it was, presumably, used liberally, and thoroughly shaken. He reports no appreciable retardation of the enzym by it unless it be a gradual weakening after long standing of fifteen days or more. His results were, therefore, similar to ours.

Van Hall (1903) used chloroform in his work upon his *Bacillus omnivorus*. His results are surprisingly at variance with those of all these others, since he found the addition of even 0.5% of chloroform destroyed all trace of activity in bacterial juices in one-fourth of an hour. We are unable to reconcile this with our experience and in view of all the evidence must believe him in error in his interpretation of results.

A COMPARISON OF THESE GERMICIDES.

The trials of the chemicals previously mentioned were conducted at about the same time and there were frequent opportunities for comparisons. Toward the close of the work a special series of trials was planned as follows in order to reach more definite conclusions upon the comparative effect of these chemicals on the activity of the enzym.

A series of ten broth cultures (10 c. c. each) five days old was treated as follows:

To each of two tubes No. 1 & 1′ added 1 c. c. of purified chloroform making 10% solutions. To each of two tubes No. 2 & 2′ added 0.5 c. c. of 2% formalin making 0.1% solutions. To each of two tubes No. 3 & 3′ crystal of thymol (about 0.05 g., i. e. 0.5%). To each of two tubes No. 4 & 4′ crystal

of phenol (about 0.5 g., i. e. 5%). Two other tubes were included in the series; one of these, No. 5, contained sterile broth in which the organism had grown for seven days and which had then been rendered sterile by passage through the Pasteur-Chamberland filter. The other, No. 6, contained a living culture of the organism, five days old. Three days later transfers made from each tube except the last proved sterility. On the same day a sterile cube cut from living carrot root was inserted into each of these tubes. Forty-eight hours thereafter the carrot tissue in tubes 1, 1′, 3, 3′, 4, 4′ and 6 were alike well softened, there being no evidence of inhibition by any of these chemicals and no greater softening in the presence of the living organisms than in these sterile tubes. The carrot in the filtrate (5) was considerably less acted upon and formalin (2 & 2′) showed still less softening. Further examination showed full softening in these latter tubes (2, 2′ & 5) at the end of nine days. Transfers at the close of the experiment proved continued sterility in all the tubes except one of those containing chloroform.

These confirmed the evidence from previous trials and led us to conclude that neither chloroform, thymol nor phenol had any inhibiting effect on the enzym; that well developed broth cultures sterilized by the addition of any of these possessed as active cytolytic properties as that in which the organisms continued alive; that formalin inhibited the enzymic activity; that filtration through porcelain reduced the enzym content.

To preclude the possibility of error because of the softening action upon the vegetable tissues of the broth itself, or of any of these chemicals, a series of control tubes was held in which carrot blocks were immersed in sterile broth without any added chemical and in similar ones in which the various chemicals were added in the amounts indicated in the above experiments. These carrot tissues in all cases remained unsoftened.

SECURING THE ENZYM BY DIFFUSION.

Our observations upon decaying vegetables have shown that the cell walls are affected some distance in advance of the invasion of the organisms. This would indicate the diffusion

of the enzym through the intermediate tissues as was observed by de Barry (1886) in the case of *Peziza* and by others with similar parasitic invasions. When this first came to our attention it suggested the possibility of testing the diffusibility of the enzym through some medium impenetrable to the bacteria, partly as confirmatory of the above explanation and partly as another method of studying the action of the enzym apart from the organisms. Before we got to the point of undertaking this, however, van Hall's (1902: 649) paper came to hand in which he describes his similar attempts and their very interesting results. In his studies upon his *Bacillus omnivorus* he employed a modification of the plan developed by Beijerinck in his studies upon the mosaic disease of tobacco. Van Hall's method consisted in growing his organism in streak cultures upon the surface of agar, then cutting off a surface layer from this, carrying the streak, and transplanting the layer to potato. In this way he secured the softening of the potato underneath the streak. In other cases he removed sterile bits of agar underlying the culture and transplanted them to sterile vegetable surfaces for trial.

We found the method outlined in the following experiment a more convenient way of securing the same result.

Beef broth agar, two per cent, was poured into small petri dishes to a depth of about 3 mm. When this had hardened and the surface dried slightly, *Bacillus carotovorus* was implanted on the surface of a small area at the center. At the end of three days a good surface growth was thus secured about 1 cm. in diameter. A slice somewhat larger than this layer of agar was then cut from the interior of a fresh turnip root and placed in a large sterile petri dish, using caution to avoid contamination. The layer of agar from the smaller dish was then carefully lifted with sterile instruments and placed upon the surface of this turnip slice in the larger dish and covered to prevent contamination. This was designated A. The details of the method may become clearer upon examination of the accompanying figure. Two other dishes, B and C, were prepared in like manner at the same time. A layer of sterile agar was laid in a fourth dish upon a turnip slice as a control and designated D.

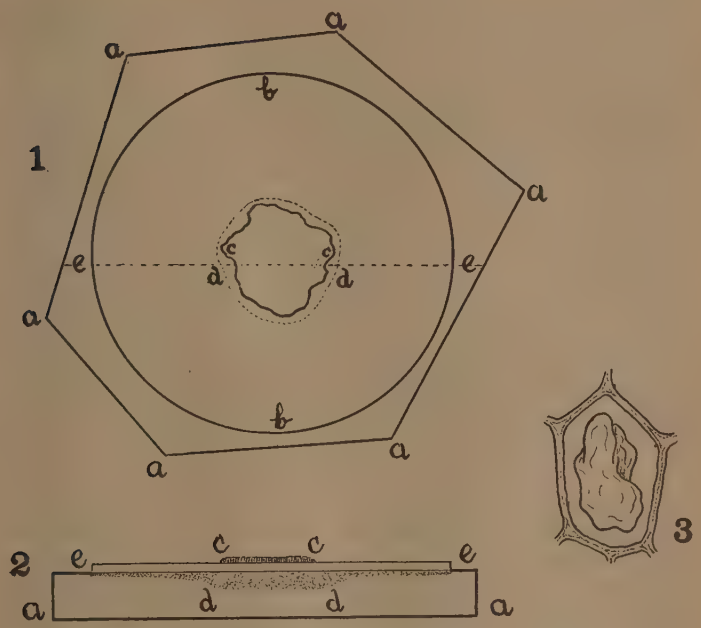

FIG. 2. DIAGRAMS TO SHOW METHOD AND RESULTS OF TESTING THE DIFFUSIBILITY OF THE ENZYM THROUGH AGAR.

No. 1, surface view; 2, vertical section of the same along dotted line e–e; a-a, sterile slice of living turnip root; b–b, layer of nutrient agar bearing the bacterial colony c–c; d–d, region of most active enzymic action; the extent of softened tissue at the end of 24 hours is indicated by the dotted area in No. 2; No. 3 shows a single cell of this softened turnip tissue.

At the end of twenty-four hours, examination of this control, D, showed the turnip underneath the agar to be unchanged, except for a slight yellowish stain imparted by the agar to the surface; no softening whatever had developed. Upon lifting the agar in the first dish, A, the turnip showed an area immediately underlying the colony and somewhat larger than the surface spread of this in which the tissues were slightly browned and softened exactly as where invaded by the organism. Bits of this rotten turnip tissue were immediately transferred to each of three broth tubes to test

sterility. In none of these, neither in any of several similarly tested later, did growth develop, showing beyond question that this softening of the tissues was due solely to the bacterial products which diffused through the layer of agar from the surface colony above. Upon more carefully examining the turnip slice it was found that there was a slight softening of the surface over the entire area covered by the agar, b-b, of the diagram Fig. 2. The area immediately underlying the colony was softened much more deeply however, viz., about 1-1½ mm. The deepest softening occurred in a circle about 2 mm. wide underlying the margin of the colony, lying between d and d in the accompanying diagram, (Fig. 2). Microscopic examination showed exactly similar conditions to those which accompany bacterial invasion, viz., isolation of the cells as a result of the solution of the middle lamellae, with the residual walls swollen and taking a blue stain upon treatment with chlor-zinc-iodide, and the protoplasmic contents showing granulation and plasmolysis.

Several other dishes examined after twenty-four to thirty-six hours show results practically like the above except that in most of them the softening was to a uniform depth of 1-2 mm. immediately underlying the colonies with a gradual decrease in this depth, as shown in the accompanying diagram which is drawn to exact scale as to size of colony and degree of softening at the end of twenty-four hours.

In connection with the above work we also conducted similar transplantations of colony-bearing layers of agar to the surface of sterile gelatin plates. Examination in such cases at the end of three days revealed liquefaction of the gelatin underlying the colony, but for an area equalling about twice the diameter of the colony above, and to a depth of one to two millimeters. Transfers from this liquefied gelatin to broth tubes proved its sterility and showed that the action here, as in the case of the vegetable tissues, was due to the diffusion of the bacterial products from the colony through the intervening agar.

SECURING THE ENZYM BY PRECIPITATION WITH ALCOHOL.

Strong alcohol added to bacterial culture broths gives a flocculent whitish precipitate which includes not only the enzyms present, and various proteid matters, but also carries the bodies of the bacteria down with it. This is the commonest method of securing enzyms in bacteriological investigations and is, therefore, in a measure a standard for comparative work. It also has advantages over the other methods especially in that it is possible easily to preserve this dried precipitate for indefinite periods. For these combined reasons it was used in much of our work. We have found that 25 per cent of alcohol is fatal to the carrot-rot organism in broth cultures and since more than that amount was used in all our precipitation work, it is evident that this method insures the elimination of the living organism.

METHODS.

Several questions arose at the beginning as to methods which we will discuss under the following heads:

1. *Filtration.*—The first of these was as to whether the alcohol should be added directly to the culture broth, thus giving a precipitate containing the bodies of the bacteria, or whether it is preferable to remove the bacteria by passing the broth through a porcelain filter before precipitation. The trial reported on a preceding page showed that the filtered broth when tested directly possesses less enzymic activity than does the unfiltered broth. As is there shown, the precipitate obtained when such broth is rendered 80% alcoholic similarly has less enzymic strength than the precipitate from unfiltered broth. After a few trials had determined these facts all subsequent work along this line was with culture broths which had simply been passed through filter paper.[4] This filter re-

[4] Some students of cytolytic enzyms have objected to the use of filter paper because of the possible action of the enzym upon it. This occurred to us early in our work but repeated observations have shown this enzym is entirely inactive on the celluloses proper. We have, therefore, used Schleicher & Schull's filter paper, both for filtering the broths and for collecting the precipitate.

moves the coarser deposits but not the bodies of the bacteria. To the filtrate was then added enough 95% alcohol to render it alcoholic to the desired degree, usually 80%, the precipitate allowed to settle, the supernatant alcohol siphoned off, the precipitate, collected on filter paper, washed with either 95% or absolute alcohol and quickly dried, partially in a current of warm air, then in a desiccator over sulphuric acid. The dried precipitate, which is gray and somewhat brittle, was then powdered before redissolving in water. It is of course important to secure quick drying to avoid the possibility of alteration as a result of bacterial growth or of chemical changes in the precipitate. The drying must also be done at so low a temperature as to preclude danger of injury from heat to the sensitive enzym. In our earlier work we washed out the 95% alcohol with absolute alcohol in order to hasten the drying. Later it was found this made scarcely any difference in the time and no difference in the result, providing the moist precipitate was properly broken up so as to dry out quickly. Spieckermann (1902: 165) used absolute alcohol followed by ether, presumably to secure quick drying. Most of our work had been completed before his paper reached us, but we thereupon tested this method in comparison with that followed by us and have found it unsatisfactory. The precipitate when the ether was used showed a diminution in its enzymic activity providing it stood in the ether long enough to displace the alcohol. Thus, holding the precipitate in ether one hour, while it did not injure it, made no appreciable difference in its rate of drying; where in ether fifteen hours, it was only two-thirds as active and required almost as long for drying; where in ether for twenty-four hours it dried quickly, but possessed only one-fourth the activity of that dried directly from 95% alcohol. While a solution of the latter softened radish and turnip tissues in fifteen minutes, the former required one hour to accomplish the same result.

In further trials chloroform was used with part of the precipitate and ether with another part to remove the alcohol with a view to hastening the drying. Thus one-half of the

precipitate was dried immediately after washing with 95% alcohol. The balance was immersed for nineteen hours in absolute alcohol, then for forty-eight hours in chloroform,[5] changing the chloroform once during this period. Upon its removal from this fluid the precipitate quickly dried to a chalky, brittle, white mass. Comparative trials, using 5% aqueous solutions of the precipitate dried from the 95% alcohol and that where the chloroform was used, showed them to be equally active. These results established our confidence in the method of drying directly from 95% alcohol and we have therefore continued so to do as it is more economical of both chemicals and time. We would again say, however, that its most successful use is in our judgment conditioned upon quick drying secured by breaking up the moist precipitate and placing it in a current of dry, warm air. Inasmuch as we found the use of ether injurious to the enzym and van Hall (1903) says that chloroform destroyed it in his trials, it is at least incumbent on anyone who employs either of them to determine their safety. It is, of course, possible that even with the same enzym secured from different broths and in mixture with different compounds there might be different results with the same chloroform or ether, and even more likely that some brands of ether or chloroform might carry in solution substances acting deleteriously upon so sensitive a compound as these enzyms.

2. *The most favorable strength of alcohol.*—Precipitation with various percents of alcohol was tried early in the work to determine the relative amounts and strengths of the precipitates thus secured.

In the first trial, using beef broth cultures five days old and increasing the alcohol at four steps, fractional precipitates were secured as follows:

At alcoholic strength of 20%, a mere trace of precipitate was secured.

At alcoholic strength of 40%, secured 15% of total precipitate.

[5] Mallinckbrodt's "M.C.W" brand.

At alcoholic strength of 80%, secured 80% of total precipitate.

At alcoholic strength of 90%, secured 5% of total precipitate.

Trial of the last three precipitates (i. e., 40%, 80%, 90%) on carrot sections showed all to have cytolytic activity, but that from the 90% had distinctly less than did the others. Between those of 40% and 80% there was no noteworthy difference. In a second trial the culture broth was divided into three lots of 150 c. c. each, and each lot treated separately as follows:

Lot 1, made 40% alcoholic, gave 0.005 g. precipitate, or 1% of total.

Lot 2, made 60% alcoholic, gave 0.065 g. precipitate, or 14% of total.

Lot 3, made 80% alcoholic, gave 0.390 g. precipitate, or 85% of total.

Trials of these precipitates on carrot and radish sections showed those from lots 2 and 3 to be of excellent activity and about alike, whereas that from 1 required twice as long to soften the sections.

Since in both of these trials the 80% alcohol secured practically all of the enzym, and this in a state of the highest activity, that strength alone was used in all subsequent alcoholic precipitation work.

3. *Reprecipitation.*—The precipitate obtained by the addition of alcohol to the broth is, of course, composed only in part of the enzym, the larger portion being presumably other proteid matter. In the hope of securing a purer state of the enzym a re-solution and second precipitation with alcohol was made as follows:

Two grams of the dry powdered precipitate obtained from beef broth cultures was added to 400 c. c. of distilled water, the solution placed on ice and frequently shaken. At the end of four hours one-half was filtered off and, since filtering through paper did not remove the undissolved precipitate, it was clarified by passing through a porcelain filter, then re-

precipitated by rendering 80% alcoholic. The balance stood twenty-four hours on ice and was then filtered through six inches of calcined sand. This cleared it up but slightly. Sufficient alcohol to make this an 80% solution was added and the precipitate collected and dried. Comparisons were then made between like solutions of this reprecipitate, of that passed through the porcelain filter, and of the original. That passed through the filter was scarcely equal to the original in strength; that passed through the sand was slightly stronger, but not enough so to be of practical consequence. Reprecipitation was therefore considered of little advantage and was not tried further.

4. *The relation of strength of solution to activity.*—When this dried alcoholic precipitate is added to water it swells promptly, but apparently only a small fraction of it is dissolved. Two questions arose early in our work: first, as to the relation of the strength of this solution to the activity of the enzym; second, as to the relative enzymic activity of (a) such solutions of the precipitated enzym and (b) of the original broth cultures from which the precipitates were secured.

To determine the first point amounts equal to 1%, 5% and 10% respectively were added to distilled water, plus chloroform, and their relative activities compared on carrot and turnip root sections. The results in all cases showed the activity to increase with strength of solution, but not proportionately. The average of several trials led to the conclusion that, with the precipitate used, it required twenty-five minutes in the 1% solution to secure as complete enzymic action as was secured in fifteen minutes in the 5% solution and in ten minutes in the 10% solution; that is to say, the relative activities of the 1%, 5% and 10% solutions stand in the ratio of 6, 10 and 15.

Our practice in all of the work here reported with alcoholic precipitates has been to use 5% solutions unless otherwise stated.

The second question is of quite as great interest, since it involves the query as to whether the enzym is or is not injured by the action of the alcohol.

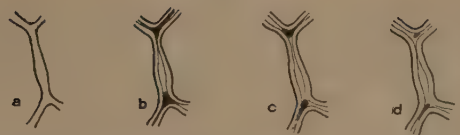

FIG. 3. STAGES IN THE ACTION UPON WALL OF THIN SECTION OF CARROT ROOT IMMERSED IN A 5 PER CENT SOLUTION OF THE BACTERIAL ENZYM SECURED BY PRECIPITATION WITH ALCOHOL.

Normal wall shown in a; b, same, 7 minutes after immersion; c, after 13 minutes; d, after 30 minutes action when only minute traces of the middle lamella remained. The cells lose coherence after the stage shown at b is reached (Camera lucida).

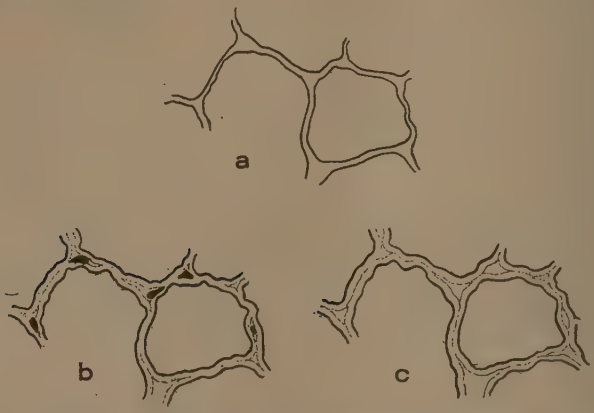

FIG. 4. A SERIES OF CAMERA DRAWINGS FROM ANOTHER SECTION OF CARROT ROOT IMMERSED IN 5 PER CENT SOLUTION OF THE ENZYMIC PRECIPITATE. The normal walls shown at a; b, the same after 12 minutes; c, the same after 32 minutes action.

In general, the beef or vegetable broth cultures yielded from 0.2 g. to 0.4 g. of the dry alcoholic precipitate for each 100' c. c. of broth. It is evident that the 1% solutions of this precipitate should contain some three times as much of the enzym as did the original broth cultures, providing that the precipitation and the re-solution are complete. On the other hand it is quite conceivable that the enzym may be injured by precipitation, in which case the aqueous solution of the precipitate might show weaker enzymic action than the original broth.

To test this matter a portion of the precipitate from a six day beef broth culture was added to a volume of water equalling that of the original broth (0.015 g. of precipitate in 5 c. c. of water, equalling 0.3% solution). The activity of this solution was then compared with that of a like broth culture sterilized by the addition of chloroform, and of third sterilized by passage through a Pasteur-Chamberland filter. The results were that the first and second showed practically the same degree of enzymic activity, whereas the third showed less than half as much. These comparative trials were repeated on a later occasion. Here we used (a) the juice expressed from a turnip decayed by *B. carotovorus,* inoculated three days previously; (b) an aqueous solution of the alcoholic precipitate obtained from a similarly decayed turnip, so made up that the volume of water equalled that in the original juice from which the precipitate came. No chloroform was added to (a), the living culture being used. Trials of these on turnip and carrot sections showed the enzymic activity of (b) to be fully as great as that of (a). The results of Spieckermann (1902: 166), which reached us after the earlier trials were made, are in accord with these. It is surprising that no loss occurs through precipitation and re-solution of a compound so sensitive as this enzym shows itself to be in many ways.

In these trials the original broths and the aqueous solutions of a strength equalling them have proved on the average less than one-third as active as the 5% solutions which we have used in most of our trials with the precipitate; that is to say, these 5% solutions have rotted the vegetable sections in less than one-third the time required to do this where the sections are immersed in the living cultures.

RELATION OF CULTURAL CONDITIONS TO ENZYM PRODUCTION.

THE MEDIUM.

The vigor of growth of the organism varies widely, of course, with the composition of the medium and with other cultural

conditions such as age and temperature. Various experiments were undertaken to determine the relation of these matters to enzym formation, having in mind especially the conclusions of some previous investigators that enzym production in certain cases is a starvation phenomenon.

De Bary (1886) looked upon the disorganization products of the enzym of *Peziza sclerotiorum,* which dissolves the middle lamella of the cell walls of various plant tissues, as the chief source of the nutrition of the fungus mycelium. He thought some sugar, available as food for the fungus, to be the product of the enzym action on the host cell walls. He considered, however, that the enzym was similarly concerned with action upon the protoplasm of the host.

Ward (1888) concluded that the similar wall-dissolving enzym produced by the lily Botrytis is a starvation phenomenon. Brown and Morris (1890) consider starvation to be a stimulus to cytase as well as diastase secretion in germinating barley. They also (1893) found that diastase formation occurs more actively in leaves during the night and concluded that this is attributable to the exhaustion at that time of soluble food, and hence is to be classed as a starvation phenomenon.

Careful comparisons have been made as to the enzym product from growths upon several media[6] varying widely in nutritive elements and especially in carbohydrate content. These have included:

1. Dunham's peptone solution (1% Witte's peptone, 0.5% sodium chloride); a medium upon which this organism makes a very weakly growth.

2. The same plus 2% cane sugar. In this the organism makes a poor growth as compared with beef broth, but the clouding is estimated to be twice as dense as in the simple Dunham's solution. This growth is of short duration, however, owing, probably, to development of acidity, which inhibits or even kills the organism.

[6] For details as to composition of the various culture media used in our work and the relative development of the organisms on these media, see Vt. Sta. Rpt., 13: 314. (1900.)

3. Neutral beef broth; the standard medium used in our work and one upon which the organism makes a good growth.

4. The same plus 2% cane sugar; a medium in which the growth is more rapid than in plain broth.

5. Cooked carrot broths. Two kinds have been used:

(a) Those in which equal weights of pieces of fresh carrot roots and of water were cooked and sterilized together by the fractional process in the steamer,

(b) The same in which, after the first cooking in the steamer, the roots were crushed and the liquid expressed and filtered through several thicknesses of paper to remove all of the cell-wall substance, then this filtrate returned to the flask and sterilized in the steamer by the fractional process. Both of these have, except in certain cases discussed below, proved to be the best of cooked media for this organism.

6. Living vegetables. Fresh living roots of carrot and turnip are both quickly invaded and rotted by this organism, furnishing apparently ideal nutritive conditions. The expressed juice from such recently decayed vegetables[7] was used in comparison with the preceding broth cultures 1-5.

The results were in general determined by comparing on razor sections of carrot and turnip roots the cytolytic action of like solutions of the alcoholic precipitates obtained from the above culture liquids.

The sixth method, using fresh uncooked vegetable, has given the most active enzym product as well as the largest amount thereof. In comparison with beef broth, which yields on an average about 0.25% of dry precipitate (i. e. 0.5 g. of dry precipitate from a 200 c. c. broth culture), this expressed juice from decayed turnip after filtration through paper has yielded over 0.5% of a precipitate, a 5% aqueous solution of which

[7] These were, of course, so handled as to insure pure growths of the organism. This was most surely and satisfactorily done by taking several roots, washing thoroughly, soaking twenty minutes in 0.1% solution of corrosive sublimate, rinsing in sterile water, then with precautions against contamination removing the surface to a depth of 0.5 to 1 cm., cutting thick pieces from the interior tissues and laying in sterile petri dishes. Inoculations on the surface of these at temperature 20°–24° secured their decay in two to three days.

caused the complete rotting of a razor section from a turnip in ten minutes, whereas a like solution of the precipitate from a beef broth culture required nearly two hours. Thus the former scheme not only gave twice as much precipitate but a solution thereof twelve times as active as was a like solution of the latter. Another comparison was made by immersing the section directly in the living cultures, i. e., in the expressed juice of decaying turnip and in beef broth cultures of the same age, viz., four days. Turnip sections so immersed were fully rotted in twenty-five minutes in the turnip juice, whereas fifty minutes was required to do this in the beef broth culture; in other words, the former was fully twice as active as was the latter. In other trials the difference was even more marked, the vegetable juice being three times as active as were the broth cultures.

The precipitates from the cooked vegetable broths, (a) and (b) of the fifth group have behaved about alike, indicating that the presence or absence of cell-wall substance has no effect on enzym production. Where a good growth has occurred, somewhat more precipitate was secured than from beef broth, which approximated but did not quite equal in weight that from the living tissues in enzymic activity. Since these were not secured and tested at the same time it is not possible to make exact comparisons. We have not, however, found these cooked vegetable media uniformly satisfactory.[8] In some

[8] We have had some puzzling experiences with such cooked vegetable broths, both of carrot and turnip. In our earlier trials (1900–01) they proved satisfactory media. In later ones (1902–03) they were unsatisfactory, this organism and various other soft-rot organisms failing to make strong growths in them. We have been forced to attribute this to the development of inhibiting poisonous compounds as a result of the cooking. It is known that cooking at high temperatures in the autoclave may develop poisonous compounds in vegetable broths which will completely inhibit bacterial growths. Dr. F. G. Novy advises us that these are probably decomposition products of the carbohydrates. We have, therefore, never autoclaved such vegetable broths, but always sterilized by discontinuous cooking in the steamer. After experiencing the above troubles we tried cooking at still lower temperatures, in one case below 80% but the results were no more satisfactory. Thinking the difficulty might be in the glass ware, we used very carefully cleaned Jena glass flasks but this seemed to make no difference. These differences are not associated with any marked loss of pathogenicity or other changes that we could detect in the organism and we were forced to attribute them to variations in the vegetable used.

cases excellent growths have developed, in others but weakly ones. In the former, as already stated, active enzymic development occurred, in the latter very little, i.e., the enzymic development was directly proportional to the vigor of growth. The beef broth cultures have given less active enzyms than these vegetable media, as has been explained. We have, however, continued to use beef broth largely in our comparative studies for the reason that the enzymic activity is sufficient for those purposes and more reliance can be placed upon the uniformity of the medium. The addition of 2 per ct. sucrose leads to more vigorous growth of the organism, especially in the earlier stages before too great acidity developed, and it is significant that more of the precipitate and with more enzymic activity was developed in the sugar broth (medium No. 4) than in the plain broth (medium No. 3).

Thus companion cultures of these two media nine days old, of which the sugar broth showed an acid reaction (+4.2 per ct.) and the plain broth was practically neutral (+0.7 per ct.), were rendered 80 per ct. alcoholic. The sugar broth yielded 0.3 per ct. of dry precipitate, the plain broth 0.25 per ct. A comparison of these cultures on carrot sections showed the former to be twice as active as was the latter, *i. e.*, to soften a like section in one-half the time. A repetition with these two media, precipitating on the sixth day, gave similar differences but a little less marked. The Dunham peptone solution has proved a very poor medium whether with or without the addition of sugar. The alcoholic precipitate from such cultures seven days old (media Nos. 1 and 2 above) have shown scarcely appreciable enzym content.

In conclusion, then, we may note that there seems a perfect correlation between the rate and vigor of the growth of the organism and the amount of enzym developed, i. e., the more vigorous the growth the more the enzym; that the presence of cell wall substance had no appreciable effect on the amount of enzym developed; and that in beef broth cultures the addition of sugar, which favors growth, also increased the enzym production.

There is nothing whatever here, therefore, to indicate that this enzymic production is a starvation phenomenon, but rather to the contrary, since the more vigorous the growth the more the enzym; moreover, the addition of carbohydrate food (sugar) seemed to stimulate enzym production, whereas the presence or absence of cell wall tissue seemed without effect. This last fact suggests the idea that the organism makes little or no use for nutritive purposes of the wall substance which it dissolves.

THE AGE OF THE CULTURE.

The enzym content was compared in carrot broth cultures (grown at 20–22° C.) of the respective ages of one and a half, three, five, seven, and nine days. A rapid increase was found from a scarcely distinguishable activity in the precipitate from the one and a half days' growth to a large amount from that of five days, and a continued but slower increase to the strongest action from the oldest cultures, viz., nine days. It was noteworthy that the increased degree of enzymic activity in the precipitates from these carrot broth cultures was accompanied by a like increase in the degree of rottenness of the vegetable tissues in the culture flasks.[9]

With cultures made in beef broth grown at laboratory temperature the results were less marked than those just recorded for the carrot broths. The outcome in the series showing most positive differences is indicated herewith. Each culture consisted of 150 c.c. of broth neutral to phenolphthalein:

[9] L. H. Jones, a student in our laboratory, working upon another (undetermined) species of soft-rot organism reached like conclusions. Thus he found that cultures in potato broth eight days old gave an enzym less than one-half as active as were similar cultures sixteen days old. While a 5% solution of the alcoholic precipitate from eight days' growth required twenty-five minutes to decompose a turnip section, like solutions from the sixteen days' growth rotted the sections in ten minutes. When the eight day culture flask was opened it was found that the potato blocks were not fully softened, but when the sixteen day flask was opened the blocks were completely rotten.

Culture No.	Age when tested.	Reaction	Amount of Precipitate.
1	3 days	—2.2%	0.495 g.
2	6 "	—1.0%	0.460 "
3	9 "	—0.9%	0.474 "
4	17 "	neutral	0.445 "

Like solutions (5 per ct.) of these precipitates tested on carrot sections showed all to contain the enzym, the activity of the solution increasing with the age of the culture. There was distinctly more in 2 than 1; the difference between 3 and 2 was very slight and that between 4 and 3 not great. Another similar series of cultures of ages four, six, nine and eighteen days respectively showed practically like enzym activities. The amount of enzym here, as in the trials with broths of different composition, seemed directly proportioned to the amount of growth. In carrot broths the growth is slower in starting than in beef broth and persists in its increase for a longer time. This slower start in comparison with beef broth is very likely due to the excess of organic acids present in the vegetable broth and to the further increase in acidity during the early development before the soluble carbohydrates are used up. In the neutral beef broth this inhibiting influence is not present and the bacterial development is at its height in about four or five days, as judged by the degree of cloudiness of the broth. Here, as in the preceding trials, there is no evidence that the enzym formation is a starvation phenomenon, but rather the reverse—viz., the more vigorous development is accompanied by more enzym production. Moreover, the enzym after its excretion into the broth appears to be a fairly stable compound, hence tends to accumulate with the age of the culture. R. E. Smith (1902) develops a theory of the parasitism of *Botrytis cinerea* somewhat at variance with Ward's idea that the enzym development is a starvation phenomenon, and more in accord with our own observations on this bacillus. Smith's conclusion is that the fungus in the absence of abundant food cannot develop the wall-dissolving enzym. It can, however, develop a toxin which kills the host tissues. He regards the initial penetration of the host tissues by spore tubes following this as

merely mechanical. Later, having acquired "vital energy" as a result of higher nutriment, the production of the wall-dissolving enzym occurs and this aids in the subsequent spread of the fungus through the host tissues.

TEMPERATURE.

The optimum temperature for most rapid growth of *B. carotovorus* is in the neighborhood of 28° to 30° C., i. e., tubes will show clouding more quickly at this than at lower or higher temperatures. The presumption would seem to be that enzym production would be most active at this temperature, but our trials have shown otherwise. Where the enzymic activity of precipitates from broth cultures grown for eight days in the incubator at a constant temperature of 30° C. were tested in comparison with those grown at room temperature (18° to 22°), the latter have shown distinctly more enzym than those grown in the incubator. We can offer no satisfactory explanation for this. Possibly comparisons at an earlier stage, say at three days' growth, would have shown somewhat different results. It is also possible that the difference is due to less aeration in the flasks held at the constant incubator temperature than occurs in those at the frequently fluctuating room temperature. It is evident that this lack of aeration would have more inhibiting influence with the older growths than with the early clouding. The result convinced us that better enzym production could be had outside than inside the incubator, and since making these trials all our cultures have been carried at room temperatures.

RELATION OF VARIOUS CONDITIONS TO THE ACTIVITY OF THE ENZYM.

EFFECT OF LONG KEEPING.

It is difficult to measure and record the rate of activity of such an enzym with a sufficient degree of accuracy to make exact comparisons. We have not succeeded in doing this to our entire satisfaction. So far as we can judge, however,

there is no loss where the dried enzym-containing precipitate is kept for months or even years. Thus two samples of precipitates prepared and carefully tested in May, 1901, were kept and again tested in May, 1903, when so far as we could judge their activity was as great as when prepared two years before.

Spieckermann (1902:166) states that the activity of the similarly dried enzym precipitate obtained from this kale-rot organism was undiminished after four months in the dry state.

RELATION OF TEMPERATURE TO ACTIVITY.

Temperature relations were studied, using solutions of the alcoholic precipitate from carrot broth cultures and testing them on carrot sections. It was found that the action was slight at 2° C., good at 22°, better at 32°, best at about 42°, inhibited somewhat at 48°, showed pronounced inhibition at 50° and was practically or entirely checked at 51° and above. For example, the action was nearly twice as rapid at 42° as at 22°; and at 32° it was practically midway in rate between the higher and the lower. The optimum lay between 40° and 45°. When such solutions were held at various temperatures up to 49° for an hour, either in the presence or the absence of carrot tissues the enzym was uninjured, i. e., they showed normal activity when the temperature was lowered again. If, however, the heating was carried to 51° or above for ten minutes, whether in the presence or absence of carrot tissues, little if any action ensued thereafter.

A comparison of these results using the precipitated enzym with those described earlier in this article where the original broth was used, shows that the points of inhibition and destruction were approximately ten degrees lower in the solutions of the precipitate.

It is interesting in this connection to recall (cf. Green 1901:448) that observations upon invertase have shown that it withstands a temperature higher by 25° C., when cane sugar, upon which it acts, is present than it does in its absence. A similar variation, though not so extreme, has been observed with several other enzyms. This suggests that, in general, an

enzym may enter into such a relation, either with the substance upon which it acts or with some other compound associated with it in solution, that as a result it may receive some protection against the injurious action of heat or other deleterious agencies. It is, of course, possible that the enzym in the original broth is in such relation to some organic matter as to be thus protected, but our attempts to protect it by the presence of carrot tissues in these experiments were unsuccessful since, as stated above, it was destroyed at the same temperature whether in the presence or the absence of the carrot sections. It is to be noted, however, that we were working here with the alcoholic precipitate redissolved in water Woods (1899) has shown that the ozydizing enzyms of the maple leaf withstand higher temperature when in the juices of the plant than when in the presence of alcohol.

In comparison with our results it is interesting to note that Brown and Morris (1890) found 35°–40° C. an especially favorable temperature for the cytolytic enzym of germinating barley, whereas it became decidedly less energetic at 50° and was almost completely paralyzed at 60°.

EFFECTS OF ACIDS AND ALKALIES.

The organism as studied was found to be parasitic on various vegetables, all of which possess an acid cell sap. In the course of its development, however, it renders the sap alkaline. It appears of interest, therefore, in connection with the question of the parasitism of the organism to learn the relation of the reaction of the medium to the activity of the enzym. This was investigated, using the alcoholic precipitate obtained from carrot broth cultures. Solutions of this were made in distilled water containing additions of the chemicals under trial and their activity tested on razor sections of carrot. The strength of the acid and alkali solutions was in all cases determined by titration against phenolphthalein.

Alkali.—It was found that the presence of sodium hydroxide titrating —2% inhibited the reaction slightly, and that the

inhibition increased with the further addition of the alkali up to —10%, where it was total.

Acids.[10] —A very slight addition of hydrochloric acid seemed favorable to the action of the enzym, a reaction of +0.5% being about the optimum. The difference between this and the neutral solution was, however, slight. When the reaction was +2.5% or above there was great inhibition and at +5% it was practically complete.

Various organic acids were tested also, the results in detail being as follows:

Acid.	Strength of titration.	Effect.
Oxalic	+0.8 percent.	retarded slightly.
"	+1.1 "	" greatly.
"	+8.0 "	complete inhibition.
Acetic	+0.2 "	no effect.
"	+0.5 "	" "
"	+1.0 "	retarded greatly.
"	+10.0 "	complete inhibition.
Formic	+0.15 "	no effect.
"	+0.4 "	" "
"	+0.75 "	retarded greatly.
"	+7.5 "	complete inhibition.
Tartaric	+0.14 "	no effect.
"	+0.55 "	" " (possibly slight retarding).
"	+5.5 "	almost full inhibition.
Malic	+0.2 "	no effect.
"	+0.8 "	" "
"	+8.0 "	almost full inhibition.
Citric	+0.2 "	no effect.
"	+0.8 "	" "
"	+8.0 "	almost full inhibition.

From these results it will be seen that these organic acids in no case aided the action; that where the acidity, as shown by titration, was +0.5% and less they were practically without effect; that +1.0% and above distinctly inhibited in all cases where it was tried, and that from +5% to +10% led to complete inhibition. Here again it should be noted that even this large amount represents only a very mild degree of acidity, viz., 0.5% more or less by weight.

[10] These acids were made up by weight and titration strength determined afterward. One per cent gravimetric solutions titrated respectively as follows: hydrochloric +50; acetic +20; tartaric +11; citric +15.5; malic +15.5; formic +15.

EFFECTS OF PLANT JUICES.

Inasmuch as in the cases of actual decay of vegetables the enzym must occur in solution in the cell sap, it is of interest to learn whether the normally acid sap tends to retard its action as do the organic acids mentioned above. Two series of trials were made to determine this. In the first the juice was expressed from living tissues of each, carrot, radish and ripe tomato. These were tested by adding to each, respectively, equal parts of a 5% aqueous solution of the precipitated enzym from a carrot broth culture. The result was, therefore, a 2.5% solution of the precipitate in half-strength vegetable juice. There was slight retardation in all cases in the rate of action as compared with solutions in distilled water, this being a little more pronounced in the case of the tomato. The test was repeated with the tomato juice by dissolving 5% of the precipitate directly in the juice, thus placing the enzym in the presence of the full degree of acidity. Here the retardation was considerable, estimated at nearly one-half, i. e., there was about as much action in fifteen minutes in a water solution as in one-half hour in the tomato juice solution.

Titration of these vegetable juices showed the acidity of the tomato to be +5%, of the carrot +2%, of the radish +0.75%.

THE EFFECTS OF OTHER BACTERIAL PRODUCTS.

As a result of his studies on the bacterial soft rot of the turnip due to *Pseudomonas destructans,* Potter (1900:451) suggested that oxalic acid produced by that organism may play some part in the destruction of the middle lamella and the separation of the cells. The above results show that neither oxalic acid nor any of the normal acids of the host tissues so function in the carrot rot organism. Indeed this organism produces no oxalic acid. It does, however, produce a small amount of some undetermined acid in the presence of carbohydrates. In order fully to determine whether this unknown organic acid or other products of the bacterial metabolism favor or retard the enzym action, broths of various kinds in

which the organism has been grown were heated to 80° C. to sterilize them and to destroy their enzym content. Two parts of each of these, respectively, was then added to one part of a solution in water of the enzym-containing precipitate and the activity of this mixture was tested in comparison with a solution of like strength of the precipitate in pure water. More or less inhibition resulted in every case, as follows: Carrot broth, cultures twelve days old, reaction to litmus slightly alkaline, slight inhibition; beef broth, cultures seven days old, reaction to litmus slightly alkaline, marked inhibition; Dunham's peptone solution, cultures sixteen days old, reaction to litmus neutral, decided inhibition, estimated to be one-half as active as the solution in pure water; Dunham's peptone solution plus 2% sugar, cultures sixteen days old, reaction to litmus strongly acid, decided inhibition, so that the tissues tested in this solution were not more acted upon at the end of twenty hours than were those in the simple Dunham's solution at the end of two hours. There is no evidence here, therefore, that the products of the bacterial growth aid in the cytolytic action of this organism. On the contrary, the evidence is that they tend to inhibit it.

DIASTATIC ACTION.

Repeated trials by the most delicate methods we could devise have failed to reveal any diastatic action worthy of note. The only indication observed has been an extremely slow and slight tendency to the conversion of starch into amylodextrin, as shown by a gradual change in iodine reaction from a clear blue to slightly purplish tint. Starch granules are not eroded even in cultures on potatoes, nor is there any change in the iodine reaction of such raw potato tissues, nor of cooked potatoes when used as a culture medium, except the slight one toward the purplish tint just noted. More delicate tests were made by mixing 1% of the washed starch from potato or wheat flour with water, heating to the boiling point, allowing to settle nearly clear and then filtering off the supernatant liquid. In this way a very weak starch solution was secured,

but one which gives a clear iodine reaction. Saliva added to this solution removes the last trace of starch in a few moments. Equal volumes of this starch solution added to a 5% solution of the enzym-containing alcoholic precipitate from a carrot broth culture of *B. carotovorus* underwent no change even after nine days' standing, other than the slight conversion toward amylodextrin noted above. The enzym-containing alcoholic precipitates from beef broth cultures were likewise inactive. In this respect, again, the carrot-rot organism differs from Potter's white-rot organism of turnip (1901) and agrees with Spieckermann's cabbage rot organism (1902).

Grüss and Reinitzer, as explained in detail later in this paper, have advocated the idea that the cytolytic action of barley malt is simply due to diastase and hold that no "cytase" as distinct from diastase occurs in such extract. Newcombe's work (1899:81) shows the incorrectness of their conclusions as regards malt extract, and we are convinced from our experiments that in this soft-rot organism we have an enzym different from diastase.

THE ACTION OF THE ENZYM ON THE HOST PLANT TISSUES.

THE COMPOSITION AND ORIGIN OF THE MIDDLE LAMELLA.

Inasmuch as the action of this enzym is chiefly upon the middle lamella of the host cells, it will make the subsequent discussion of this matter clearer if we briefly review the facts as at present understood relating to the composition and origin of this portion of the cell membrane. Fortunately, some excellent work along this line has been done within recent years.[11]

Examination of any mature parenchymatous cell, as of carrot or turnip root, shows the middle lamella as a more or less clearly defined refractive line through the middle plane of the cell walls. On either side, i. e., lying between this and the cell cavity is an inner lamella, or, as Allen calls it, "primary wall." Where three or more cells meet in mature tissue inter-

[11] See critical reviews of the subject by Green (1901:298–300) and Allen (1902).

cellulars commonly occur and often slits or openings radiate from these for some distance. These result from mechanical strains, doubtless caused by growth changes.

Where such openings occur it is usually evident that the middle lamella substance has split along its middle plane and a brightly refractive border line of this substance bounds each inner lamella externally along these intercellular slits and spaces. In many cases, however, no such intercellular has developed and the middle lamella substance extends as an apparently homogeneous layer of slightly varying thickness between the adjacent inner lamellae of the walls. At the junction of three or more cells this expands into an angular mass, completely filling the space formed by the meeting of their rounded contour lines. As seen in section these masses are most often triangular. There is some evidence from their deeper staining properties that these are denser than the thinner lamellar plates, and our observations on the rate of solution, to be discussed later, are in accord with this idea.

Various opinions have been held as to the composition and origin of the middle and inner lamellae of the walls. It has long been understood that parenchymatous walls of the kind under discussion are composed of cellulose. More recently it has been shown that "cellulose" includes a group of closely related compounds. Moreover, if cellulose stains or solvents be applied to parenchymatous tissues it will appear that the inner lamellae are fundamentally cellulose, whereas the middle lamella does not give the cellulose reactions. More critical observations will show that the inner lamellae are rarely if ever homogenous but also contain substances other than cellulose.

Cross and Bevan (1895:78, 89) in their discussion of celluloses make two groups, (1) the cellulose group, (2) the compound celluloses. They further sub-divide the cellulose group into three sub-groups:

(a) Resistant to hydrolysis, e. g., cotton.

(b) Less resistant to hydrolysis, found in grass stems, etc.

(c) Low resistance to hydrolysis, found especially in fleshy roots and in seeds.

They term groups (a) and (b) the celluloses proper. Group (c) is held to be quite different from these, but for convenience of treatment they allow it to remain in the larger group, accepting Schultze's name as satisfactory, viz., pseudo-cellulose or hemicellulose. As defined more exactly the hemicelluloses are "substances closely resembling in appearance the true celluloses but easily resolved into simpler carbohydrates by the hydrolytic action of enzym or of the dilute acids or alkalis."

We are, however, chiefly concerned with the compound celluloses which Cross and Bevan term "pecto-celluloses," since these constitute the middle lamella and other wall elements acted upon by the carrot-rot enzym. The present understanding of these dates from the work of Fremy (1840, 1848), who found in plant cell walls, along with cellulose, another substance which he called pectose. He also isolated from carrot roots and other plant tissues an enzym "pectase" capable of gelatinizing this pectose and related compounds, which will be discussed in more detail later. Subsequently chemists have confirmed Fremy's observations and conclusions and class the pectose series of compounds with the celluloses as indicated in the discussion above from Cross and Bevan. Mangin has recently (1888–1893) made most extensive studies upon these matters and shows that here again we have not a simple compound but a complex of closely related compounds. These he divides into two natural series, the one neutral, the other acid. Pectose is one of the less soluble neutral series, and pectine is a more soluble form. Both of these are of wide distribution, especially in the walls of young tissues. Of the acid series pectic acid is of common occurrence and peculiar interest to us and especially its insoluble salt calcium pectate. Fremy supposed that when his enzym, pectase, clotted the pectose solutions it did so by converting the pectose into pectic acid. Bertrand and Mallevre (1894, 1895) have recently shown that this clot is, however, calcium pectate. Payen [12] believed that the middle lamella consists largely if not wholly of this salt, and the recent studies of Mangin and Bertrand-Mallevre

[12] Cf. Green 1901:248.

have confirmed this belief. Moreover, these recent studies have shown that the inner lamellae contain varying proportions of pectose or pectic compounds intermingled with the celluloses. The relation of these is evidenced if Schweitzer's reagent, which is a cellulose solvent, be carefully applied, when it will remove the cellulose and leave the pectic skeleton. The converse occurs, as will be shown in detail later, when the carrot-rot enzym acts upon the walls, removing the pectic elements and leaving the cellulose.

Mangin's studies led him to conclude that in the early stages of its development the wall consists more largely of the less soluble pectose, whereas in the mature wall the calcium pectate predominates in the original plane, i. e., the middle lamella, and the pectose which occurs is in the inner lamellae, i. e., nearer the cytoplasmic layers. The proportion of cellulose becomes increasingly predominant, however, as one passes from the middle to the inner layers. Although this is the case, there probably occurs, even in the young walls, a thin sheet of calcium pectate invisible under the microscope but evidenced by the splitting of the walls along the middle plane under the action of pectate solvents. With the increasing age of the cell this layer is thickened and more clearly defined until it becomes plainly visible in the mature cell as the middle lamella. The splitting of the lamella along the median plane as a result of the tensions set up between the growing cells indicates that this apparently homogeneous plate is in reality from the beginning a double sheet, one-half of which originated with each daughter cell following mitosis.

Fremy's enzym, pectase, which is especially abundant in growing tissues, is supposed to function [13] in this lamella formation by converting the insoluble neutral pectose of the inner lamellae into the more soluble pectine and ultimately into pectic acid, which then passing, perhaps by diffusion pressure, to the outer surfaces of the inner lamellae, i. e., to the planes where this meets the middle lamella layers, is there combined with calcium to increase this middle lamella substance. This appears homogeneous, but as will appear later is, like the inner

[13] Cf. Green. 1901:297–300.

lamella, distinctly stratified in structure, at least in the heavier parts at the angles.

This conception involves the idea of the passage in the growing walls of the pectose substances by gradual filtration through the cellulose layers from the protoplast where they originate toward the exterior, and is at the same time in accord with the idea of Allen (1902:31) that the young cambium wall really forms the basis of the middle lamella of the older tissues.

THE ACTION OF THE ENZYM ON THE CELL-WALL.

The rapidity of the invasion of vegetable tissues by the carrot rot organism was discussed in our earlier report (1900:307–312). As there explained it rots only parenchymatous tissues. The invaded tissues become watery and usually more or less darkened in color when exposed to the air. The cells rapidly lose all coherence and always show a sharply defined line of demarkation, indicating that the softening occurs quickly and completely after it begins. Examination of such recently decomposed tissues under the miscroscope shows the cells to be already isolated or easily separable along the plane of the middle lamella. The protoplasmic sac within the cell is collapsed, more coarsely granulated than normally, and evidently dead and in the process of disorganization. Bacteria teem around and between these cells but are so rarely seen within them that where this does occasionally occur, one is led to attribute it to mechanical rupture of the softened walls rather than to direct solution. (See figures 1, 5 and 6.)

In the case of the inoculation of a cut surface of root kept in ordinarily dry atmosphere, the invaded area dries out very rapidly; if, however, it is kept in a saturated atmosphere gray drops of exudate teeming with bacteria form on the surface and the tissues underneath become sunken. It is evident, therefore, that among the products of the bacterial growth are active osmotic substances which draw the water, and of course soluble nutritive matters, from the dying or dead protoplasts. The organism is powerless to invade wilting or pithy and partially dried-out vegetable tissues of even the most susceptible varieties, such as turnip, radish and carrot. These facts, with

others to be set forth later, show its active invasion to occur in the intercellular spaces and along the planes of the middle lamellae. A fundamental condition of this invasion is an abundant moisture content in the host tissues, the more the better, apparently. The water-logged, or translucent appearance of the invaded tissues is doubtless due in part to the expulsion of gas incident to the filling of the intercellulars with liquid resulting from the plasmolysis of the cells, and in part to the changes in the optical characters of the walls themselves. The fresh walls are uniformly refractive throughout with a slight difference between the middle and the inner lamellae. Almost immediately following their immersion in either a living culture or an aqueous solution of the precipitated enzym the inner lamellae begin to lose their refractiveness. This change in appearance is evident even to the unaided eye if thin sections are closely observed. It is more rapidly followed under the microscope, and is then seen to be associated with a swelling of the primary wall or inner lamellae, sometimes to twice their original thickness, and with the appearance within a short time of a delicate laminated structure in these swollen walls, as shown in the accompanying figures. The middle lamellae

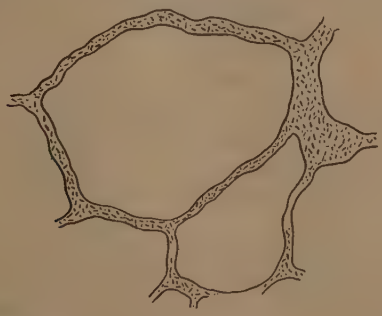

FIG. 5. A sterile block cut from living carrot root was immersed for 20 hours in a broth culture of *B. carotovorus*, then fixed in hot absolute alcohol, imbedded in paraffin and sectioned. The above sketch (x 250) shows a large thin-walled parenchyma cell lying about three cells inward from the surface. The enzymic action was here complete, the cells isolated and the intercellular spaces gorged with bacteria, but the undissolved remnant of the wall kept them from invading the cell cavities.

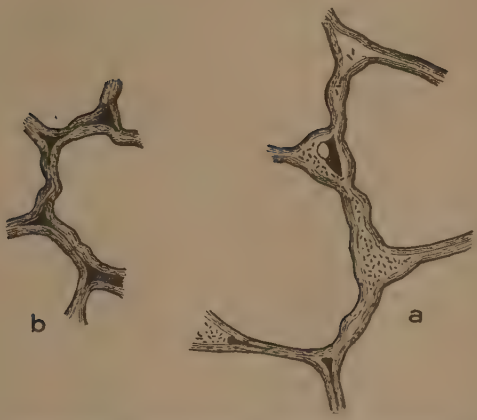

FIG. 6. From deeper lying portions of the same sections as Fig. 5. (x 375). The cells here were smaller and thicker walled. In a the bacteria were abundant in the intercellulars and the middle lamellae partially dissolved; b, from a little deeper, showed less advanced action, the walls swollen and laminated but still cohering.

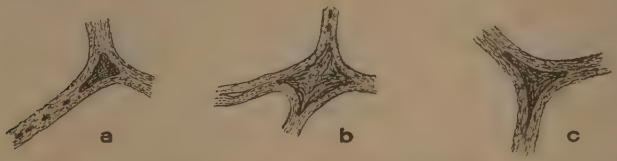

FIG. 7. Cell membranes from the central part of the same sections (x 500). The earliest stages of bacterial invasion along the plane of the middle lamellae are shown in a and b, while c shows the action upon the walls in advance of invasion. The laminated structure appears both in the swollen inner lamellae and in the undissolved portions of the middle lamellae which occur at the angles of the cells.

also become less refractive, though not softly translucent, as do these adjacent inner layers, and soon begin to melt away in the thinner portions. The middle lamellar substances, as already explained, usually form thicker masses at the angles of the cells, often triangular as seen in optical section. As the thinner parts dissolve, these heavier portions remain isolated. There are now distinct openings between the adjacent cells in most

places and the swelling of the walls has reached its maximum. Tapping on the cover glass, tearing with needle point or other mechanical test, will show that the cells have lost all cohesion, that is, that the tissues are fully "rotten." Thin razor sections of carrot or turnip placed in living cultures or in active enzym solutions pass through these changes in from ten minutes to an hour, although longer immersion may be necessary to secure the complete solution of the thickest pieces of intercellular substance. Meanwhile there is a slight thinning of the walls proper from without inwards, i. e., from the lamellar side towards the cell cavity, but this is not great and complete solution has never been observed either in the presence of the living organisms or in solutions of the enzym. In order to determine this matter to our satisfaction the same sections have been kept under observation for three weeks, with repeated measurements and camera drawings during this period, but there was little change after the first few hours. Cellulose stains (iodine and sulphuric acid or chlor-zinc-iodine) give clear blue reactions with these fully softened walls, and this reaction is the same even after the longest immersion. The lamination of the walls becomes increasingly apparent for a short time, after which there is no further change.

In no case have we found evidence of any action whatsoever upon lignified or cuticularized walls. Vessels lie for days or weeks in the presence of the enzym with the walls unchanged in refractive character or other appearance or in staining reaction to phloroglucin.

In order to follow the course of invasion more carefully blocks of fresh carrot tissue cut from near the core, with precautions to insure sterility, have been immersed in broth culture tubes of *B. carotovorus* and at varying periods of time after the surface tissues had begun to show decomposition they have been carefully transferred to absolute alcohol to kill and fix the organisms in place, then imbedded in paraffin and sections cut three to twelve microns in thickness. For differentiating the wall tissues and organisms various stains have been tried,

including Ziehl's carbol-fuchsin and aqueous solutions of Congo red, ruthenium red and methylene blue.

The best results were obtained from the use of ruthenium red and methylene blue. It was found that about two minutes' immersion in each of these in turn, followed by brief washing in alcohol, gave slides showing excellent differential staining. The organisms, the unaltered walls and the undissolved remains of the middle lamellae retained the blue color and in the walls which had been fully acted upon the red color predominated. Examinations of slides so stained have shown very interesting conditions. The Congo red also clearly reveals the solvent action, staining deeply the unaltered walls and giving but faint color to those from which the soluble part has been removed. The line of demarkation between such deep and faint staining tissue is very abrupt, indicating that the solvent action is rapid and complete after the penetration of the active substance. This action occurs some ten cells, more or less, in advance of the invasion of the organism. It is evident that the chemical agent causing this change penetrates the tissues and completes its action considerably in advance of the invasion of the organism. The rate of the invasion was clearly dependent upon the nature of the walls and the form of the cells. As already stated, no action occurs on cuticularized or lignified walls. Where the cells were much elongated in one direction the action progressed more rapidly in the direction of their longer axis. The organisms in the more recently invaded portions were chiefly found in the larger intercellular spaces at the angles of the cells. From these they made their way, evidently along the planes of the dissolved lamellae, occurring in the narrower portions as a single line of rods lying end to end. In no case were they within the cell cavities, although they often crowded the lumina of the open vessels. The walls themselves showed in these stained sections the same transitions noted in the fresh material, viz., swelling, and evident lamination of the inner lamellae preceding the full solution of the middle lamella. The middle lamellar substance itself, especially at the angles of the cells where it occurred in larger

masses, showed distinctly a laminated or fibrillar structure when partially acted upon, indicating that it, like the wall, is not of entirely homogeneous structure.

The discussion thus far has been based upon observations made upon carrot tissues. Studies of the invasion of turnip and radish roots and cabbage petioles have shown practically similar conditions. The rate of softening of sections of these tissues in solutions of the enzym-containing precipitate and in living cultures has proved to be more rapid in the turnip, radish and cabbage tissues than in those of the carrot. In the latter the action was faster on the core than on the cortex tissues. It was more rapid in the young potato than in the mature tuber. On the beet root no action whatever occurred.

In order to secure data for the above conclusions careful trials were made on two occasions with a 5% solution of an alcoholic precipitate containing a not very active enzym. This weaker enzym, acting more slowly, permitted more satisfactory differentiation between the rate of softening of tissue of similar susceptibility. The trials were made about July, using vegetables fresh from the garden except for the potatoes of Series I. The details are as follows:

Series I. Using thick razor sections of (1) old potato, core; (2) young carrot, a. core, b. cortex; (3) young radish, core; (4) young turnip, core; (5) cross sections of young cabbage petiole. The interval before complete disintegration was: turnip, forty minutes; radish and cabbage, about forty-five minutes; carrot core, eighty minutes; carrot cortex, ninety minutes; potato, one hundred minutes.

Series II. Using similar solutions and vegetable sections, except that sections from a young potato tuber fresh from the garden were substituted for the old potato (1), and the following were added: (6) cotyledon of pea, approaching maturity; (7) root of beet. The intervals before complete disintegration were: turnip, radish, cabbage (about alike), thirty-five minutes; young potato, carrot core (about alike), eighty minutes; carrot cortex, pea (about alike), one hundred minutes; beet, limp but no signs of disintegration even after twenty-four hours' immersion.

These observations as to the rate of action of the enzym on plant tissues clearly accord with the results from inoculations

into the corresponding vegetables made in our earlier studies (1900:307) and indicate, what we would expect, that aside from moisture relations the relative susceptibility or resistance of the host plants to infection depends largely, if not wholly, upon the composition of the middle lamellae.

A COMPARISON OF THE ENZYM PRODUCED BY BACILLUS CAROTOVORUS, WITH CYTOLYTIC ENZYMS FROM OTHER SOURCES.

CYTOLYTIC ACTION BY SOFT-ROT BACTERIA FROM OTHER SOURCES.

In the course of these studies upon the cytolytic enzym of the carrot-rot bacillus comparisons have been made with the various other strains of soft rot organisms described in Part I of this bulletin, p. 258. In order to make this clear we will summarize the list, which includes 45 strains, as follows:

Three strains of cabbage-rot bacilli isolated in Vermont by F. R. Pember in 1899.

Twenty-three other strains of cabbage-rot bacilli isolated in Vermont by W. J. Morse in 1901.

One strain of turnip-rot bacillus isolated by L. P. Sprague in Vermont, 1903.

Twelve strains of soft-rot bacilli secured by Harding and Stewart in New York, of which one was associated with the soft rot of *Amorphophallus simlense* and the other eleven were from the soft rot of cabbage.

Six other soft-rot organisms from various sources, as follows: Townsend's calla rot, *Bacillus aroideae;* Harrison's cauliflower rot, *Bacillus olereaceae;* van Hall's two iris-rot organisms, *Bacillus omnivorus* and *Pseudomonas iridis;* Spieckermann's kale-rot organism (*Bacillus*), and Potter's turnip-rot organism, of which the strain we had was also a *Bacillus.*

Forty of these forty-five strains, with three others, were studied* in detail by Messrs. Harding and Morse, the details

*Their studies did not include the following: Pember's R., *Pseudomonas iridis,* nor the New York organisms O. 1 II 6 c, O. 1 II 6 a, and the bacillus from Amorphophallus.

of their studies being related in Part I of this bulletin. It will suffice here to say that their comparative studies as there recorded lead them to conclude (p. 287) that these forty strains probably constitute only one somewhat variable species. The following comparisons as to enzym production were, however, completed before their decision was reached and we believe they were worth recording partly as contributing further evidence as to the general likeness of these strains and partly as emphasizing the minor variations which we believe must always be expected to occur with different bacterial strains, even of the same (so-called) species.

The comparisons as to enzym production have been made by testing both the living cultures and the alcoholic precipitates, following the general methods outlined earlier in this article. Inasmuch as the alcoholic precipitate yields the enzym in a more concentrated form, and one in which it can be preserved indefinitely for comparative trials, this method has been chiefly relied upon. In all cases much care has been exercised to insure uniformity in the several series as to medium and cultural conditions. The trials of cytolytic activity were made on razor sections of vegetables, carefully selected for uniformity, and the trials repeated until convincing evidence was obtained as to relative activity. Inasmuch as it was not practicable, for obvious reasons, to make close comparison of more than a limited number of these at a time, they were handled in series of six or eight in a group. Since there was some variation in the vegetable upon which the test was made in different cases it is to be noted that *B. carotovorus* of the original strain, or some other organism whose activity was well known, was included in each series. In all cases except Pember's organism R cytolytic action occurred. In order to have a basis for comparison the organisms of each series were grouped in order of cytolytic activity into three classes, as follows:

Class I. Activity moderate, *B. carotovorus* being the standard of this class.

Class II. More active than I, intermediate between I and III.

Class III. Most active, Vermont XLVIII and Turnip Rot D well representing this class.

The following tables give the results of the trials grouping the various organisms in these classes according to cytolytic activity.

SERIES I. EIGHT DAYS' GROWTH; CULTURAL CHARACTERS ALIKE IN ALL; CYTOLYTIC ACTION TESTED ON CARROT AND CABBAGE.

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
Calla rot (B. aroideae)[14]	1%	.465 gr.	111.	0.
Pember A.....	1%	.254 "	111.	
" R.....	1%	.532 "	0.	
Vermont XXXIII....	1%	.344 "	11.	
" XLVIII....	1%	.370 "	111.	
B. carotovorus.....	1%	.467 "	1.	0.

[14] In some other trials Calla rot has proved about like *B. carotovorus* in enzymic activity.

SERIES II. NINE DAYS' GROWTH; CULTURAL CHARACTERS ALIKE IN ALL; CYTOLYTIC ACTION TESTED ON RADISH.

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
Pember A.....	1.1%	.355 gr.	111.	0.
Vermont XLVIII....	1.4%	.374 "	11.	0.
" LI....	1.3%	.391 "	1.	0.
" LIV....	1.4%	.393 "	11.	0.
" LVI....	1.2%	.434 "	1.	0.
" XCIV....	1.3%	.366 "	1.	0.
" XCVII....	1.1%	.293 "	111.	0.
" CII....	1.4%	.260 "	11.	0.

SERIES III SEVEN DAYS' GROWTH; CULTURAL CHARACTERS ALIKE IN ALL; EXCEPT THAT MORSE'S 98 SHOWED LESS AND PEMBER'S 2 HEAVIER CLOUDING THAN THE REST; CYTOLYTIC ACTION TESTED ON RADISH AND TURNIP.

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
Vermont XXV....	1.%	.300 gr.	11.	0.
" XXIX....	1.%	.241 "	11.	0.
" XXXI....	1.1%	.241 "	1.	0.
" XXXIII....	0.7%	.344 "	11.	0.
" XCVIII....	0.9%	.275 "	11.	0.
" CIII....	1.%	.185 "	11.	0.
Pember C.....	0.8%	.366 "	111.	0.
" R.....	1.3%	.350 "	0.	0.

SERIES IV. SEVEN DAYS' GROWTH; CULTURAL CHARACTERS ALIKE IN ALL: CYCTOLYTIC ACTIVITY TESTED ON TURNIP AND RADISH.

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
Vermont XLVIII....	1.1%	0.315 gr.	111.	0.
" XLIX....	1.2%	0.335 "	11.	0.
" L....	0.8%	0.386 "	11.	0.
" LII....	0.4%	0.230 "	111.	0.
" LV....	1.%	0.317 "	1.	0.
" XCV....	2.%	0.304 "	111.	0.
" XCVI....	1.2%	0.300 "	11.	0.
" XXVI....	1.2%	0.303 "	11.	0.

SERIES V. SEVEN DAYS' GROWTH; CULTURAL CHARACTERS ALIKE IN ALL; CYTOLYTIC ACTIVITY TESTED ON TURNIP AND RADISH. (VERMONT XLVIII FROM SERIES IV WAS INCLUDED IN HERE AS A CONTROL.)

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
Vermont C.....	1.3%	0.334 gr.	11.	0.
" CI.....	1.2%	0.238 "	111.	0.
New York, 0.2 e.....	1.2%	0.410 "	1.	0.
" Miller, 3 No. 2	0.9%	0.380 "	111.	0.
" 0. R. Be.....	1.%	0.334 "	1.	0.
" Miller 2, No. 2	0.9%	0.252 "	11.	0.
" 0. R. Bi.....	1.2%	0.400 "	111.	0.
" 0.1 II 6 c....	1.%	0.440 "	11.	0.
Vermont XLVIII.....	1.1%	0.315 "	111.	0.

SERIES VI. SEVEN DAYS' GROWTH; CULTURAL CHARACTERS ALIKE EXCEPT GENEVA 10 WHICH SHOWED HEAVIER CLOUDING AND PELLICLE; ENZYMIC ACTIVITY TESTED ON TURNIP AND RADISH.

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
New York, Miller 3, 3....	1.3%	0.361 gr.	1.	0.
" Miller 3, 1...	1.5%	0.240 "	11.	0.
" Riverhead 3, 1	1.7%	0.250 "	11.	0.
" 0.1 II 6a....	1.3%	0.134 "	1.	0.
" 0.2 f.....	1.5%	0.264 "	111.	0.
" Riverhead 2, 1	1.8%	0.237 "	11.	0.
Vermont XLVIII.....	1.5%	0.258 "	111.	0.

SERIES VII. SEVEN DAYS' GROWTH; CULTURES SIMILAR IN VIGOR AND GENERAL APPEARANCE; TESTED ON TURNIP.

Organism.	Reaction.	Weight of Precipitate.	Cytolytic activity.	Diastatic activity.
B. carotovorus.....	1.4%	0.689 gr.	1.	0.
Kale rot (Spieckermann)	2.%	0.626 "	111.	0.
Turnip Rot D.....	1.8%	0.674 "	111.	0.
B. olereaceae.....	1.7%	0.350 "	1.	0.
B. omnivorus.....	1.8%	0.435 "	11.	0.
Potter's bacillus.....	2.%	0.676 "	11.	0.

These results are in general accord with those of preceding investigators so far as published.

Potter describes (1899, 1900) for his *Pseudomonas destructans* action upon the wall like that we have observed for *B. carotovorus*. He records later (1902:393) evidence of direct penetration of the softened cellulose remnant of the wall by the organism. This is probably to be regarded as due to physical pressure rather than to solution of membrane, though

it is so interesting and important a thing that it is to be hoped that further confirmatory evidence may be adduced. Potter also records diastatic action by this organism. In comparing our results with his it is to be borne in mind that the organism we have in culture is probably not his original organism, since this is a *Bacillus* instead of a *Pseudomonas.*

Spieckermann (1902) describes in detail cytolytic action by his organism identical with that observed by us, and absence of diastatic action.

Van Hall (1903) likewise describes in detail cytolytic action stopping short of complete solution of the cellulose wall and absence of diastase action by *Bacillus omnivorus.*

Harrison (1902) has attributed solution of the middle lamella to the organisms described by him.

We are therefore convinced that in all of these cases, including the forty-five strains of organisms from different sources, there is developed the same middle-lamella-dissolving enzym as in *B. carotovorus,* and that moreover in all cases alike there is neither complete solution of the cellulose elements of the wall nor diastatic action.

CYTOLYTIC ACTION AS RECORDED FOR OTHER BACTERIA.

The fact that the softening and solution of plant cell walls result from certain bacterial growths has been known for many years.

Mitscherlich in 1850 observed the destruction of cell walls and consequent liberation of starch when potatoes decompose in water, and believed it due to the vibriones which develop in the liquid.

Van Tieghem (1879) studied the decomposition of various vegetable tissues. He considered this due to the action of a single polymorphous species of *Bacillus* to which he gave the specific name *amylobacter.* He found that these organisms could decompose only the younger or less resistant tissues. Old tissues and those lignified, cuticularized, or suberized were resistant to the action as also was the cellulose of bast fibres.

De Bary (1887:101) accepted van Tieghem's account of the breaking up of cellulose membranes by a "diastatic enzym" in the process of decay attributed to *Bacillus amylobacter*. It is, however, generally agreed by bacteriologists to-day that this name is applicable to a class of bacteria rather than to any single species.

Vignal (1889) in his monograph on *Bacillus mesentericus vulgatus* records that it secretes a ferment capable of dissociating the cells of potato tubers by dissolving the intercellular substance, i. e., middle lamella, but without dissolving the cellulose of the wall. Similar action occurred upon parenchymatous tissues of beans, chestnuts, turnip, carrot, cabbage, beets and numerous young stems when these were immersed in the cultures. In none of these did full solution of the cellulose walls occur even after three months. It is noteworthy that this organism at the same time developed several other enzyms, viz., diastase, a proteolytic enzym, and a rennet, and that the cellulose remnant of the wall persisted in the presence of all of these.

Heinz (1889) observed a similar disorganization of the tissues of hyacinth as a result of the invasion of the tissues by the organism he describes as *Bacillus hyacinthus septicus*. It is noteworthy that he found this organism incapable of liquefying gelatin, in which it differs from the carrot-rot organism.

Van Senus (1890) observed the solution of fibrous and parenchymatous plant tissues by bacteria. He attributes the action to *B. amylobacter* and a smaller kind acting conjointly, neither alone accomplishing it. His methods have been considered by later investigators too crude to insure reliable results.[15]

Kramer (1891) isolated from decaying potatoes an aerobic spore-forming bacillus capable of dissolving the intercellular substance of potato tissue, and of attacking the cellulose membrane also.

Frank (1899) found dissociation of the cells of potato as a result of the invasion of *Micrococcus phytophthorus*. The action of this organism likewise stopped short of the solution of the

[15] Cf. Omelianski, 1902:200.

cellulose layers, removing only the middle lamella, and also left the starch grains intact.

Wehmer (1898) studied the bacteria concerned with the rotting of potatoes, all of which he regarded as of saprophytic nature. He found two types of decomposition to occur, associated with different organisms. In the first, "breifäule," the middle lamellae only are dissolved. He considers that the acid produced by the bacteria may be the agent in this solution of the pectic compounds, rather than an enzym. In the second, "schleimfäule," there is ultimately solution of the entire wall substance.

Laurent (1899) working with *Bacillus coli communis* found that, although normally a saphrophyte as concerns plant tissues, yet when inoculated into weakened vegetables it developed there and in so doing acquired virulence as a parasite upon potatoes of full vigor, and also upon turnip and onion. There was no secretion of diastase (amylase). Heating the culture to 62° C. for five minutes destroyed this ferment.

Lepoutre (1902), continuing Laurent's work by similar methods, developed strains pathogenic to plant tissues of three other species of bacteria, normally saprophytic, viz., *Bacillus fluorescens liquefaciens, B. mycoides* and *B. mesentericus.* The first acted like *B. coli communis* in Laurent's cultures, viz., dissolved the middle lamella, but not the cellulose or the starch. Lepoutre considered the solution of the lamella due to an enzym which he speaks of as a variety of pectinase, evidently accepting the name suggested by Bourquelot and Herissey (see discussion later in this paper).

Migula (1900:529) states that the culture of his *Bacillus asterosporus* upon slices of cooked carrot leads to the solution of the middle lamella.

Winogradsky (1895) studied the retting process of flax and considered it due to a single specific anaerobic bacillus. He concluded that this fermented the pectin elements readily but was without influence on cellulose proper, e. g., Swedish filter paper. This conclusion is in accord with the opinion expressed by Kolb (1868) that the retting process is essentially a pectic fermentation.

Behrens (1902) studied further the organisms concerned with the different methods of flax retting. He found that in all cases alike the essential thing is the solution of the middle lamellae of the parenchyma cells. He concluded that in the latter process a specific anaerobic bacillus is the agent, while in other processes fungi (Mucors) take the active part.

Haumann (1902) studied the flora of retting flax and concluded that there are numerous species of bacteria and fungi active in the process. He found several of these species capable of liquefying calcium pectate.

Doubtless further search would reveal other records of the solution of the middle lamella without evidence of the solution of the cellulose layers. It may safely be inferred, in our judgment, that the action in most of these cases was like that in the soft-rot organisms studied in our laboratory. These citations will at least suffice to emphasize the point that such action on middle lamellae apart from action on cellulose is a common occurrence in bacterial fermentation. It must not be concluded, however, that cytolytic action by bacteria is confined to the middle lamella and like parts of the wall. While this is the action concerning which we have the most detailed evidence, proof is not lacking that there are bacteria capable of causing the full solution of parenchyma walls, and even of the more resistant cellulose of fibre.

E. F. Smith (1903) has shown this in his painstaking work upon the black rot of the cabbage caused by *Pseudomonas campestris*.

No other equally convincing evidence has come to our attention as to like action by bacteria parasitic upon plants. We have no doubt, however, that such cases will be found not infrequently as bacterial plant diseases are more fully investigated.

Omelianski (1895) began in 1894 a study of the solution of normal or typical cellulose as represented by Swedish filter paper. The final summation (1902) of his painstaking work shows that at least two species of bacteria are capable of causing the complete solution of this most resistant cellulose. He

believes that further study will reveal other such organisms. He considers that in the so-called "cellulose-fermentation," earlier writers were dealing chiefly or wholly with the more easily hydrolyzable celluloses and pectic compounds.

CYTOLYTIC ACTION OF CERTAIN FUNGI.

Although it has long been known that fungi penetrate cell walls, de Bary (1886) first separated a cytolytic enzym from the living fungus and studied its characters and action. He thus proved that *Peziza sclerotiorum* (*Sclerotina libertiana*) secretes a soluble ferment which causes some swelling of the walls of vegetables invaded by it, followed by a solution of the middle lamella and consequent isolation of the cells. He observed a partial solution of the inner lamellae, and the residual portion gave a beautiful cellulose reaction with chlorzinc-iodide.

Ward two years later (1888) did his painstaking work on the lily *Botrytis*. Here he found an enzym which causes a swelling and lamination of the inner and solution of the middle lamella of the cell walls of the host plant. He considered it "extremely probable this ferment is of the same nature as the one extracted by de Bary." Ward also observed the extension of cytolytic enzym drops from the tips of the *Botrytis* hyphae, which he considered to be associated with the hyphal growth.

Arthur (1897:499) records similar lateral exudations upon the young hyphae of *Rhizopus*, which he believes to be associated with the local secretion of cytohydrolytic enzym.

Laurent (1899) in subsequent studies upon the *Sclerotinia* enzym found it to be destroyed by heating to 54° C. when in solution, whereas it required 62° C. to destroy the middle lamella dissolving enzym of *Bacillus coli communis*. He concludes from this that these enzyms must be different. His argument is less convincing from the fact that his two enzyms were secured from cultures made on different vegetable media, viz., artichoke in one case and potato in the other. There may have been by-products from the *Sclerotinia* culture which lowered its resistance. The fact was referred to, earlier in this paper, that Green (1901:448) and others have recorded much

wider differences, under varying conditions, in the thermal destruction point of certain other enzyms. This must at least make one cautious in accepting conclusions drawn from such evidence.

Kissling (1889) studied the pathogenic action of *Botrytis cinerea* and found that it formed a poison which kills the protoplasm of the invaded tissues, and which he thought enzymic and the same as the ferment secured by de Bary from Peziza. Kissling did not, however, differentiate between the two changes which occur, viz., the death of the protoplasm and the dissociation of the cells.

Kean (1890) showed that the decay of sweet potatoes and other vegetables as a result of the invasion of *Rhizopus nigricans* (*Mucor stolonifer*) is due to an enzym which softens the tissues, but he does not give the details of the changes as secured under the microscope. He found a similar softening by the juice expressed from potato leaves infested with *Phytophthora infestans,* also from a *Botrytis* growing on stone crop (*Sedum*).

Miyoshi (1895) observed the penetration of the walls of different plants by *Botrytis* and *Penicillium* and inferred the presence of an enzym, but did not isolate it.

Behrens (1898) published the results of his extensive studies upon the pathogenic action of *Botrytis vulgaris* (*B. cinerea*)[16] upon fruits and clearly distinguished between the toxic and cytolytic action. He showed that there is a toxin developed which is not destroyed by boiling, thus confirming de Bary's observations, and in addition an enzym or enzyms which acted like de Bary's in causing dissociation of the cells. He found enzymic action from *Penicillium glaucum* and *P. luteum,* but only upon the middle lamella. In order to determine the relation to cellulose and calcium pectate respectively he grew these fungi for forty-eight days on culture fluids containing Swedish filter paper. He found that *Botrytis* reduced the total solids whereas these increased with the other two. He concludes that *Botrytis* is capable of dissolving cellulose, probably

[16] Smith, R. E., shows these names to be synonyms.

converting it into dextrine, whereas the others are not. He then cultivated all three upon a synthetic medium containing 1% calcium pectate. He found that these fungi grew better upon this than upon a similar medium containing arabinose instead of the pectate. On the other hand *Oidium fructigenum* did better on the arabinose medium. This result accorded with his later observations that the first three dissolve the middle lamella of host plants by enzymic secretions, whereas the *Oidium* cannot, but makes its way wholly by mechanical pressure. He is of the opinion that the enzym of *Penicillium,* which dissolves the middle lamella but not the cellulose, is different from the cellulose-dissolving enzym.

Nordhausen (1899) at about the same time made, independently, similar studies upon *Botrytis cinerea* and reached very nearly the same conclusions. He emphasizes the fact that this *Botrytis* differs somewhat in its action from Ward's lily fungus in that the enzymic solution of the middle lamella is not accompanied as a rule by the strong swelling of the inner or cellulose layers. In this respect the action is more like that of de Bary's *Sclerotinia.* He considered the swelling observed by Behrens, of the cell walls of *Symphoricarpus,* an exceptional thing for this fungus.

R. E. Smith, in a recent paper (1902) on *Botrytis cinerea,* reports that while it dissolves the middle lamella of lettuce parenchyma it causes no swelling of the residual layers. Moreover, he found the action occurred even after the fungus extract causing it had been heated to the boiling point. He considered that this action is probably identical with that of de Bary's *Peziza,* but that in Ward's lily fungus and Potter's turnip bacterium an enzym was produced different from any ordinarily produced by *Botrytis cinerea.* He emphasizes the importance of the role of oxalic acid, which is formed by this fungus and also by de Bary's.

Grüss attributed the cytolytic action of *Penicillium* to diastase. He applied the name alloölysis to such action in which the enzym evidently penetrates the substance and the solution is preceded by visible changes, forming a "corrosion zone," as

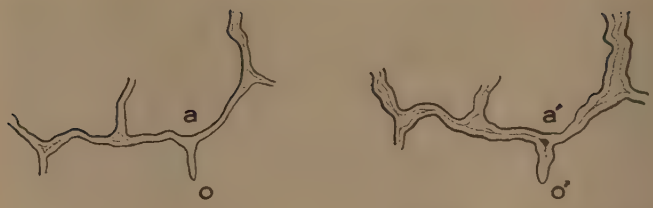

FIG. 8. Camera drawings of margin of a very thin section of carrot root tissue; a, normal; a^1, same 13 minutes after immersion in 5 per cent. solution of enzymic precipitate of *B. carotovorus*. (See also Fig. 4 where same solution was used). Since the projecting wall fragment at o^1 was most fully exposed to the cytohydrolytic action it was carefully measured to determine whether it would undergo any shortening. The enzym solution was renewed, with the addition of chloroform to inhibit bacteria, and the slide kept under observation for three weeks, but no further solution occurred.

FIG. 9. Similar camera drawings of a bit of the cell wall projecting from the margin of a thin section of carrot-root tissue. The normal wall is shown in a; b, the same after 20 minutes action of 2.5 per cent. aqueous solution of Taka diastase; c, after 22 hours action, showing the gradual solution of all parts, the middle lamella being more persistent.

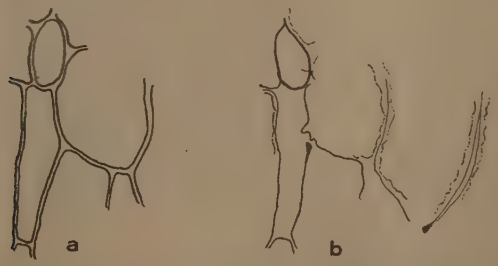

FIG. 10. Similar drawings from another preparation; normal carrot walls shown in a; b, the same after 2 hours action of 2 per cent. solution of Taka diastase. The continous lines show the more persistent middle lamellae. The inner lamellae were entirely dissolved in places.

contrasted with the surface erosion ("Abschmelzung") of starch grains by diatase.[17]

Newcombe (1899) published the results of studies upon the commercial preparation, "taka-diastase," which is the enzym-containing precipitate from the fungus *Aspergillus oryzae*. His tests upon barley endosperm sections showed a cytolytic action. The walls became hyaline, first near the middle, this change then progressing through the inner lamellae toward the cell lumina. Following this, these wholly hyaline walls (inner lamellae) began to disappear from the borders (cell lumina) toward the middle of the wall so that a faint and thin middle lamella often persisted for forty-eight hours after the beginning of the experiment. A similar melting away of the walls of the aleurone layer occurred more slowly. Newcombe also studied its action on the cell walls of the cotyledon of *Lupinus albus* with like results, the middle lamella again proving more resistant to this enzym than did the inner lamellae.

We have repeated Newcombe's work and found the results as he describes. Trial was then made of taka-diastase in comparison with the enzym from *B. carotovorus* on carrot-root walls. The results are shown in the accompanying figures 8–10.

THE CYTOLYTIC ACTION OF POLLEN-TUBES.

Elfving (1879) and Strasburger (1884) have studied the development of pollen tubes and found that in general they burrow through the plane of the middle lamella, thus passing between the cells rather than through them.

Miyoshi (1894) found the tubes capable in some cases at least of actually penetrating the walls. Both Miyoshi and Green (1894) conclude that the action of pollen tubes is such that the secretion of a cytolitic enzym must be inferred. Green made a series of careful experiments with pollen both before germination and during the process planned to demonstrate the presence of this and other enzyms which he judged to be present. He secured inulase and diastase but was unable to

[17] See abstract of Grüss article, *Centralbl. f. Bakt.* II. 2:585 (1896).

get evidence of cytolytic action from the extracts of any pollen he tested.

No later trials have come to our attention and we are therefore unable to go further than to make inferences as to the presence of a cytolytic enzym in pollen. Green concludes that it is there in spite of his failure to secure it and that it is similar to the lamella-dissolving enzym of Ward's lily *Botrytis.* The penetration of the walls observed by Miyoshi may easily have resulted from mechanical pressure without the complete absorption of the cellulose layers of the walls.

CYTOLYTIC ACTION IN SEEDS AND OTHER STORAGE TISSUES OF THE HIGHER PLANTS.

The endosperm of seeds has the cell membranes characteristically of the easily-hydrolyzed or hemicellulose type, and the same is true of other plant storage tissues such as fleshly roots and tubers. The solution of these in the normal processes of germination or growth-resumption has long interested plant physiologists.

Sachs (1862) observed the solution of the endosperm of the date palm, *Phoenix.* This was attributed to an enzym, but attempts at isolation of a soluble ferment from the seeds of another palm, *Livistonia,* by Green (1893:94) failed and it remained for Newcombe (1899:67) to secure the cytolytic enzym from germinating seeds of *Phoenix dactylifera.*

The first enzym of this class isolated from seeds was, however, obtained by Brown and Morris (1890) from barley malt. They found that the alcoholic precipitate of malt extract contained a cytolytic enzym in addition to diastase. This functions, during normal germination, leading to the solution of the endosperm walls preceding the solution of the starch granules by the diastase. The details are of interest: First, a slight swelling of the inner lamellar walls, bringing out evidence of stratification; second, the gradual solution of the modified inner lamellae, the middle lamella being the most resistant; third, the solution of the middle lamella. Newcombe (1899:52) repeated and confirmed their observations later and

states that the inner lamella was dissolved down to the middle lamella within five to fifteen hours, whereas the latter persisted for from one to ten days before complete solution.

Brown and Morris (1890:500), testing the barley enzym on other tissues, found potato sections rapidly decomposed. It was found that the cell walls swelled, became differentiated into very thin laminae which later broke up into spindle-shaped fragments and ultimately disappeared with the exception of a thin layer representing the middle lamella. The artichoke, carrot and turnip behaved like the potato, but beets and apples were affected little or not at all. Heating to 60° C. rendered the enzymic solutions inactive on the walls, whereas this diastatic action withstood 70°.

Brown (1892) has since found a like enzym in oats and rye and reached the conclusion that the cytolytic action which occurs in the early stages of the digestion of these grains by animals is due to the action of the ferment present in the grains themselves rather than to the digestive juices of the animal.

Gardiner (1897:106) observed solution of the walls in the endosperm of *Tamus communis* during germination. The disorganization of the walls was accompanied by marked stratification and the middle lamella was dissolved first, the inner lamellae later.

Grüss (1896) in working upon germinating barley, observed the same changes as Brown and Morris, and applies the name "alloölysis" to the behavior. His use of the term was explained earlier in this article. He speaks of four sorts of "diastase" which may be differentiated in germinating barley, (1) translocation diastase, (2) secretion diastase, (3) glukase, (4) cytase. He holds that the last of these "must yet be held as questionable," concluding that "secretion diastase" may cause the cytolytic action and may be more and more weakened by heating above 50° C., so that it loses the power previously possessed of acting on saccharo-colloids. It is of interest to note that Grüss' use of the word "cytase" in the above connection marks the origin of this word so far as we have learned.

Reinitzer (1897) likewise refused to admit that the cytolytic action in barley is due to another enzym than diastase, although he considers that an enzym, cytase, differing from the barley enzym may occur in seeds having the walls thickened with hemicellulose as a reserve material.

Bourquelot and Herissey (1898) made trial of the enzymic action of a barley malt extract, secured by the method of alcoholic precipitation, upon a solution of pectine from gentian. They found evidence of the presence of an enzym, destroyed by heating to the boiling point, which was capable of so changing pectine that it cannot thereafter be gelatinized by the action of the clotting enzym, pectase. This is, they believe, due to the conversion of the pectine into reducing sugars. For this enzym they propose the name pectinase.

Green (1901:104) considers that they did not prove this "pectinase" to be different from the cytolytic enzym of Brown and Morris, since the French observers did not determine what constituent of the wall is affected by it. Green appears, however, to have overlooked a later publication by Bourquelot (1899:567) in which he states that when the solution of pectine was coagulated by pectase and this coagulum treated with the malt enzym solution it gradually disappeared and coincidently reducing sugar was found, showing that it was a process of hydrolysis. He considers the action of these two enzyms, pectase and pectinase, on the compounds of the pectic series analogous to that of rennet and trypsin, respectively, on casein, in that one causes coagulation of the soluble forms while the other liquefies the coagulum through hydrolysis. As confirmatory of his conclusions he cites experiments wherein he added the two enzyms simultaneously to the pectine solution. When the proportion of the pectase was larger as compared with the amount of pectinase there resulted first coagulation and then the gradual liquefaction of the coagulum. When the proportions were reversed no coagulation took place. These latter experiments would seem to disarm any suspicion that the solution of the clot as reported in the first experiment was due to bacterial development. It is, however, a matter of regret

that Bourquelot gives practically no details as to his methods in these experiments, since it is evident from his statements that the changes occupied considerable lengths of time, and one would wish full assurance of the absence of bacterial growth.

In some of his other work with enzym solutions he relied upon chloroform, in some on "thymol water," both of which, we have learned, must be used with painstaking, if bacterial growths are to be suppressed with certainty. In some cases he has depended upon frequently raising the temperature of the solutions to 50° C. to destroy bacteria.

In spite of these criticisms, however, it seems to us that the conclusions of Bourquelot and Herissey must be accepted, viz., that there is present in barley malt an enzym, their pectinase, which hydrolyzes pectine, and also an enzym, presumably the same, which hydrolyzes the pectic coagulum. Since this latter is regarded by most chemists, including Green, as calcium pectate and the same as the middle lamella in composition, the name pectinase becomes applicable to the middle lamella-dissolving enzym of barley malt.

The strongest objection to this dictum is that there may be insufficient ground for their conclusion that the action upon the pectine and the coagulum is due to one and the same enzym. These are closely related compounds and it seems to us their conclusion must be accepted until the contrary is proved. There is nothing in their publication to indicate whether or not they regarded their "pectinase" as capable of causing the solution of the hemicelluloses also. Since, however, they do not state to the contrary and later name only three enzyms as occurring in barley malt[18] (diastase, trehelase and pectinase), we are led to infer that they so regarded it, and therefore, as Green says, they did not clearly define their "pectinase" as different from Brown and Morris' cytolytic enzym, for which Grüss later proposed the name "cytase."

Newcombe (1899) made a comparative study of cytolytic enzyms in the course of which he repeated and verified the

[18] Bourquelot, Em. Sur l'hydrolyse des polysaccharides par les ferments soluble. *Jour. Pharm. et Chem.* **16**:581. 1902.

observations of Brown and Morris upon the barley enzym and secured similar soluble ferments from some other germinating seeds. He found the enzymic extract from the cotyledons of *Lupinus albus* to be strongly cytolytic and but feebly amylolytic. Extracts from both the cotyledons and the endosperm of *Phoenix dactylifera* showed cytolytic and amylolytic activity, and here again the cytolytic action was relatively greater. There was, however, a noteworthy difference between the two date-seed extracts in that the one from the cotyledons had much greater amylolytic strength and relatively weaker cytolytic activity. This showed that the action on starch and the action on cell wall were distinct and independent processes. This evidence was further strengthened by comparisons of these with barley malt extract and with taka-diastase, both of which were found relatively stronger in amylolytic action. This seems conclusive as against Reinitzer's contention that the cytolytic action was due to diastase.

Further comparisons showed also a lack of correlation between the rate of solution of the middle lamella and inner lamellae, respectively. Thus the five solutions were made to such a strength that all showed a like rate of action on starch. Sections of barley endosperm were then immersed in them and the periods necessary for complete solutions of inner and of middle lamellae, respectively, in each found to be as follows:

Source of extract.	Solution of inner lamella.	Solution of middle lamella.
Lupinus cotyledon	required 9 hours.	required 21 hours.
Date endosperm	" 9 "	" 118 "
Date cotyledon	" 21 "	" 118 "
Taka diastase	" 94–116 "	" over 312 "
Barley malt	" 94–116 "	" 312 "

These figures also serve to indicate the differences in the relative amylolytic and cytolytic activities discussed above.

Green in the second edition of his work on Fermentation (1901:105) cited these observations of Newcombe's as confirming his earlier idea that "there appear to be two varieties of

cytase, one attacking most readily the middle lamella, the other the layers deposited upon it."

It only remains to refer in this connection to the accounts of two other enzyms of the cytolytic class.

Effront (1897) obtained from the carob bean a ferment he calls caroubinase. This acts upon a peculiar carbohydrate caroubin, differing from both starch and cellulose, which he obtained not only from this bean but also from barley and rye. Caroubinase has been classed with the cytases, but differs from those previously discussed in that it withstands a temperature as high as 80° C. It is of interest in the present discussion only as suggesting that other classes of cytolytic enzym may be revealed upon further investigation.

Wiesner (1885) states that there occurs in gum arabic an enzym capable of transforming cellulose into gum and mucilage. This apparently needs further study before acceptance, but it again suggests the same idea.

CLASSIFICATION AND NOMENCLATURE OF THE CYTOLYTIC ENZYMS.

In the first place we would again call attention to the fact that these studies have added strength to the argument that the cytolytic enzym or cytases stand in a class apart from the amylolytic enzyms or diastases. This is evidenced by the uniformly lower point of thermal destruction of the cytases; by the fact that cytases occur which show no diastatic action whatever, e. g., that of *B. carotovorus;* by the further fact that diastases occur which show no cytolytic action whatever, e. g., that of saliva.[19] Where enzymic solutions show both cytolytic and amylolytic activity they have been found to exercise these in unlike ratios, sometimes one and sometimes the other predominating, as shown by Newcombe. The only conclusion permissible is that in such cases two enzyms occur in mixture in varying proportions.

As to the cytolytic enzyms, we are convinced that Green is right in his conclusion that these fall into two natural groups,

[19] Cf. Brown, H. T., 1892:356.

the one active upon the pectic, and the other upon the cellulose elements of the cell membrane. Since both of these elements include a complex of chemical compounds we would naturally expect a corresponding variety to be shown in the enzyms which act upon them. There is, indeed, evidence that this does occur. The fuller understanding of the chemistry of the cell membranes must, however, precede such further subdivision of the enzyms acting upon each. For the present we can at least accept the following as representing well-defined groups of components in the simpler or less modified plant tissues: 1. True celluloses. 2. Hemicelluloses. 3. Pectic compounds. In the more modified tissues there are other compound celluloses, ligno-cellulose, etc., which do not here concern us.

Evidence is not lacking that there are enzyms capable of hydrolyzing the true celluloses, but further study is necessary before their relationships can be defined. The cytolytic enzyms, which have been studied in sufficient detail so that we can characterize them, act only upon the last two of these three classes. As already indicated, we consider these enzyms to be as clearly separable into two groups or kinds as are the wall elements upon which they act, and we believe it must conduce to clearness of understanding if a distinct name be accepted and defined for each of these kinds of enzym.

The enzym of *B. carotovorus* and the related soft-rot bacteria is a good example of one acting upon the pectic compounds, but not hydrolyzing the hemicelluloses. Such an enzym has heretofore been referred to usually as "cytase." If the cytolytic enzyms are to be differentiated some more specific name must be found. Following the custom in naming enzyms, pectase would be the right name had it not been applied to Fremy's clotting enzym. If one accepts the grouping of the celluloses outlined by Cross and Bevan and considers these pectic elements of the wall to occur in compound with cellulose or pecto-celluloses preference might be given to the name *pectocellulase.* This is not free from objection, however, since their term pectocellulose was applied to a hypothetical compound made up of non-hydrolyzable cellulose elements and pectic

elements which pass into solution under the influence of this enzym. This name if adopted would suggest an enzym capable of action on both these components alike.

All things considered, we favor the name *pectinase,* which was suggested by Bourquelot and Herissey, as already explained. An objection to this name is that it was originally applied to the enzym which hydrolyzes pectose. Later it was found that this same extract hydrolyzes the coagulum, or pectic clot. It was inferred that this latter action is due to the same enzym as the former. Although this was not demonstrated, it seems to us sufficiently probable to justify its acceptance as a satisfactory working hypothesis, and if so this name must be accepted for the enzym under discussion. As more broadly defined, then, *pectinase* is capable of hydrolyzing pectose when in solution so that it will no longer yield a clot under the influence of pectase, and also of hydrolyzing the pectic coagulum and the pectic elements in the cell wall, viz., the middle lamella and parts of the inner lamellae of certain tissues. As a further justification of the acceptance of the name pectinase in the broader sense we note a tendency in this direction in certain writings which have appeared since its promulgation.[20]

Bourquelot and Herissey did not so define their enzym as to exclude its action on hemicellulose; in fact, the barley malt solution with which their work was done does so act. As already explained, this hydrolytic action on hemicellulose predominates in the cytolytic action of taka-diastase, although action on the pectic compound occurs also. Since we have made some observations upon this taka preparation we will base our discussion on that. Two explanations are available for action such as occurs here, and in like cases where there is solution of both of these wall elements. The first is that only one kind of cytolytic enzym is present, which is allied to pectinase but differs from it in that it acts primarily on hemicellulose and to a less degree on the pectic elements. The second is that two distinct cytolytic enzyms are present in mix-

[20] See use by Lepoutre (1902), also Oppenheimer (1901:193).

ture, viz., a small amount of pectinase, which causes the hydrolysis of the pectic elements, and a relatively larger amount of another enzym which acts on the hemicelluloses. Newcombe's results, referred to earlier in this paper, strongly favor the second of these two possible explanations and exclude the first.

Accepting the conclusion that there is an enzym other than pectinase in taka-diastase, barley, malt, etc., of which the hydrolytic action is chiefly or wholly on the hemicellulose elements of the cell membrane, we need a distinctive name for that also. The one introduced by Oppenheimer (1901:187), cellulase, seems fit except that it is too general. It implies an activity on the celluloses generally, and especially on the celluloses proper. This enzym, which acts only on the hemicellulose, may better be termed *hemicellulase*. This is a self-explanatory name and leaves the name cellulase to be applied either in a more general sense to all cellulose enzyms, including this hemicellulase, or, as would be preferable in our opinion, reserving it for application to the enzyms which hydrolyze the celluloses proper, as recently studied by Omelianski (1902). These terms, pectinase and cellulase, have heretofore been used rather vaguely as synonyms of Grüss' term cytase, i. e., as applicable to cytohydrolysts generally. If they be restricted to the more exact usage defined above, it leaves the words cytohydrolyst, or better cytolyst, and cytase as convenient and satisfactory terms for use in the broader sense to include in a general or indefinite way both of the above, and indeed any other related enzyms capable of hydrolyzing the cell membranes.

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